



Interactions between *Campylobacter* and the human host

Nielsen, Lene Nørby

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Interactions between *Campylobacter* and the human host

PhD Thesis by:
Lene Nørby Nielsen

Department of Veterinary Disease Biology
Faculty of Life Sciences (LIFE-KU)
University of Copenhagen
&
Department of Microbiological
Surveillance and Research
Statens Serum Institut



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Preface

This thesis is submitted as partial fulfilment of the requirements for the Ph.D. degree. The work was carried out at Department of Disease Biology, Faculty of Life Sciences, University of Copenhagen and Department of Microbiological Surveillance and Research, Statens Serum Institut. The work was carried out from August 2006 to July 2010 with Professor Hanne Ingmer, Professor Karen A. Krogfelt and Postdoctoral fellow Christina S. Vegge as supervisors. The work was partly funded by the Research School for Biotechnology (FOBI).

My sincere thanks go to my supervisors for their supervision, enthusiasm and especially the freedom to explore my own ideas throughout this project. I am thankful for the opportunity to visit and collaborate with leading scientist all over the world. It gave me invaluable knowledge and contribution to this project and without the support, this would never have been possible. I am thankful for the stay in Martin Maidens laboratory at Oxford University and the great hospitality they showed me. Thanks to Samuel Sheppard for guiding me in the laboratory and also for the help that continued after my stay. Thanks to all at Nobilon in Holland for allowing me to visit and follow their vaccine study and animal experiments. Thanks to Thomas Luijckx for the kindness during the visit and after my stay. And finally I am grateful for the stay in Michael Konkels laboratory, Washington State University, where I obtained invaluable knowledge of the pathogenesis of *C. jejuni*. Thanks to all in the Konkel lab for the hospitality, also after working hours, and making the stay very special.

Thanks to all at the Department of Veterinary Disease Biology for creating an inspiring environment and especially “Campygruppen” for fruitful discussions. Thanks to Birgit Groth Storgaard for always being positive and supportive. At Statens Serum Institut I would like to thank all for a fantastic working and social environment. Thanks to Steen Stahlhut and Nadia Boisen for making the office enjoyable.

Last, I would like to thank my parents and sister for always supporting me and putting up with me throughout the PhD, my parents-in-law for making ends meet especially during the final hectic time, and thanks to Edin and Hanna for being patient, supporting and loving.

Lene Nørby Nielsen,
Frederiksberg, July 2010

List of abbreviations

Aa	Amino acid
AAG	Autoagglutination
AFLP	Amplified fragment length polymorphism
BSA	Bovine serum albumin
C.	Campylobacter
CC	Clonal complex
CadF	Campylobacter adhesion to fibronectin
CapA	Campylobacter adhesion protein A
CDT	Cytolethal descending toxin
CFU	Colony forming units
Cia	<i>Campylobacter</i> invasive
CPS	Capsular polysaccharide
DC	Dendritic cell
DNA	Deoxyribonucleic acid
<i>E.</i>	<i>Escherichia</i>
ECM	Extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
Fn	Fibronectin
FBS	Fetal bovine serum
FlpA	Fibronectin-like protein A
Ig	Immunoglobulin
JlpA	jejuni lipoprotein A
LB	Luria Bertani agar
GBS	Guillain-Barré syndrome
Kn ^R	Kanamycin resistant
LOS	Lipooligosaccharide
MAP	Mitogen-activated protein
MLST	Multilocus sequence typing
MLEE	Multilocus enzyme electrophoresis
MOMP	Major outer membrane protein
ND	Not determined
ORF	Open reading frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase chain reaction
PEB1	Protein Pei, Ellison and Blaser 1
PEP4	Protein Pei, Ellison and Blaser 4
PFGE	Pulse field gel electrophoresis
PRR	Patters recognition receptor
QS	Quorum sensing
RA	Reactive arthritis
SSI	Statens serum institut
ST	Sequence type
TLR	Toll like receptor
WHO	The World Health Organization

Abstract

Infections with *Campylobacter jejuni* are the leading cause of food borne bacterial gastroenteritis in most developed countries. The symptoms associated with campylobacteriosis range from mild enteritis to severe diarrhoea, and secondary symptoms can occur, including reactive arthritis and Guillain-Barré syndrome. *C. jejuni* pathogenesis is not fully understood but previous studies have shown that the bacterial surface is crucial for the virulence of this organism. In this thesis, we have focused on *C. jejuni* and its interaction with the human host.

In the first study, multilocus sequence typing (MLST) was used to determine the diversity and population structure of *C. jejuni* isolates from Danish patients and to determine the association between multilocus sequence typing types and different clinical symptoms, including gastroenteritis, Guillain-Barré syndrome and reactive arthritis. 51 sequence types (STs) were identified within 18 clonal complexes (CCs). ST-21, ST-45 and ST-22 were the most frequent clonal complexes isolated and the GBS isolates grouped significantly into ST-22 clonal complex. There was no significant clustering of the reactive arthritis isolates.

In the second part, we screened an expression library of NCTC 11168 *C. jejuni* genes for highly immunogenic proteins using antibodies raised in rabbit against a clinical isolate of *C. jejuni* serotype 2. This identified 52 highly reactive clones, which represented 25 different genes after sequencing. We examined some of them as vaccine candidates and for possible relation to virulence. Particularly one protein showed promising results as vaccine candidate in a mice immunization experiment and another protein could be important for adhesion of *C. jejuni* to epithelial cells and therefore human host interaction. The library was subsequently screened for genes expressing proteins involved in virulence-associated phenotypes, such as autoagglutination, biofilm-formation and adhesion to epithelial cells. Several interesting genes were identified but further work needs to be done on this part.

In the last part, we studied a putative fibronectin binding protein (Cj1349c). Fibronectin (Fn) is a glycoprotein present in the regions of cell-to-cell contact in the gastrointestinal

epithelium and a potential binding site for pathogens. We investigated the protein, which was both expressed and studied in *E. coli* and expressed and purified from *E. coli*, for its ability to bind Fn using an ELISA assay. The assays revealed a binding of Cj1349c to Fn and we suggests that the Cj1349c protein has a role in attachment to host epithelial cells via Fn binding.

In conclusion, population structure of Danish isolates was diverse but there was a significant grouping of GBS isolates. Furthermore, a possible new vaccine candidate, a putative new virulence factor and a new Fn binding protein were identified in this study.

Dansk resumé

Infektioner med *Campylobacter jejuni* er den mest almindelige årsag til bakterielle fødevarerelaterede infektioner i industrialiserede lande. Symptomerne spænder fra mild til alvorlig diarré og følgesygdomme som Guillain-Barré syndrom og reaktiv artrit kan opstå. Viden om *C. jejunis* patogenese er ikke fuldt ud forstået, men tidligere studier har vist at bakteriens overflade er afgørende for organismens virulens. I denne afhandling har vi fokuseret på *C. jejuni* og dens interaktion med den humane vært.

I det første studie brugte vi Multilokus sekvenstypning (MLST) for at bestemme diversiteten og populationsstrukturen af *C. jejuni* isolater fra danske patienter og for at bestemme associationen mellem multilokus sekvenstyper og forskellige kliniske symptomer, inklusiv Guillain-Barré syndrom og reaktiv artrit. 51 sekvenstyper (STer) blev identificeret fordelt på 18 klonale komplekser (KKer). ST-21, ST-45 og ST-22 blev isoleret hyppigst og GBS isolaterne grupperede sig signifikant i ST-22 klonal kompleks. Der var ingen signifikant gruppering af reaktiv artrit isolaterne.

I den anden del screenede vi et ekspressionsbibliotek af NCTC 11168 *C. jejuni* gener for meget immunogene proteiner ved at bruge antistoffer produceret i en kanin mod et klinisk isolat af *C. jejuni* serotype 2. Det resulterede i 52 meget reaktive kloner der repræsenterede 25 forskellige gener efter sekventering. Vi eksaminerede nogle af dem som vaccinekandidater og for en mulig relation med virulens. Specielt et protein viste lovende resultater som vaccine kandidat og et andet protein kunne have betydning for *C. jejunis* fasthæftelse til epitelceller og derved værtsinteraktion. Biblioteket blev efterfølgende screenet for proteiner involveret i virulens-associerede fænotyper som autoagglutination, biofilmdannelse og fasthæftelse til epitelceller. Flere interessante gener blev identificeret, men mere arbejde mangler på denne del.

I den sidste del studerede vi et muligt fibronectinbindende protein (Cj1349c). Fibronectin (Fn) er et glykoprotein i regionerne af celle til celle kontakt i tarmepitelet og derfor et potentielt bindingssted for patogener. Vi undersøgte proteinet, der både blev udtrykt i *E. coli* samt udtrykt og oprenset fra *E. coli*, for dets evne til at binde Fn ved at bruge et ELISA assay. Assayet viste at Cj1349c bandt til fibronectin og vi

foreslår at proteinet Cj1349c spiller en rolle i fasthæftelsen til epitelceller via Fnbinding.

Populationsstrukturen af danske isolater var divers, men der var en signifikant gruppering af GBS isolater. Ydermere blev en mulig ny vaccinekandidat, en ny mulig virulensfaktor samt et nyt fibronectinbindende protein identificeret i dette studie.

Aim and outline

The aim of this thesis was to study the interactions between *Campylobacter* and the human host, by 1) study the population structure and clustering of *C. jejuni* isolates from enteritis and sequela, and 2) identify new genes involved in *C. jejuni* virulence.

A general introduction to *C. jejuni*, its epidemiology and pathogenesis is given in chapter 1. An brief overview of the experimental work is given in chapter 2, followed by the obtained results presented in four separate chapters. Chapter 3 contains a paper of multilocus sequence typing and clustering analysis of *C. jejuni* clinical isolates. Chapter 4 and 5 contains results obtained by screening an *E. coli* open reading frame library of *C. jejuni* genes by using a novel method with antisera against *C. jejuni* (manuscript 2) and by using different virulence associated assays (manuscript 3). Chapter 6 contains manuscript 4 where results of a fibronectin binding protein are described. Chapter 7 is a general discussion of the work presented in thesis and suggestions for future research. Finally is a conclusion of the main results obtained. All references, including those cited in the paper and manuscripts, are found in the references at the end of the thesis.

1. Introduction

1.1 Taxonomy, biology and epidemiology

1.1.1 Taxonomy

In 1886, Theodor Escherich identified a spiral shaped bacteria in stool sample from neonates with diarrhoeal and this was the first description of *Campylobacter* (Skirrow and Butzler, 2000). In 1906, two British veterinary surgeons reported the presence of several novel “Vibrio” species in a range of animals and in 1947 Vincent and colleagues isolated the organism (Vincent et al., 1947). *Campylobacter* were initially classified as *Vibrio* spp. due to spiral morphology, but Sebald and Véron postulated in 1963 a new genus, *Campylobacter* (Sebald and Véron, 1963). The genus contains 15 species and six subspecies and belongs to the epsilon class of proteobacteria, which also comprises *Helicobacter*, *Arcobacter* and *Wolinella*. For the species *C. jejuni*, the focus of this thesis, two subspecies are recognized; *C. jejuni* subsp. *jejuni* and subsp. *doylei*. Little is known about the pathogenic role of the latter and in this thesis *C. jejuni* will refer to *C. jejuni* subsp. *jejuni*.

1.1.2 Biology

C. jejuni are curved or spiral rods that are 0.2 to 0.8 μm wide and 0.5 to 5 μm long. They are gram negative and do not form spores. The cells are highly motile with a polar flagellum at one or both ends of the cell. They are catalase and oxidase positive and urease negative. Temperature range for growth is 30-44°C (van Putten et al., 2009), with an optimum temperature of 42°C. This probably reflects an adaptation to the intestines of birds. *C. jejuni* are microaerophilic, requiring an oxygen concentration of 3-15% and a carbon dioxide concentration of 3-5%. They have relatively small genomes (1.6 – 2.0 megabases) and can establish long term associations with their hosts (Parkhill et al., 2000).

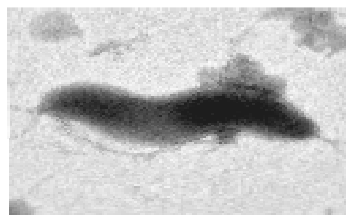


Figure 1.1. Electron micrograph of *C. jejuni*. The bacteria on the picture are the *C. jejuni* NCTC 11168 strain used in this thesis (picture taken by Christina K. Johnsen, Dpt. Virology, Statens Serum Institut).

1.1.3 Epidemiology and typing

C. jejuni is the most common cause of bacterial-mediated diarrhoeal disease worldwide and The World Health Organization (WHO) estimates that app. 1 % of the Western Europe population will be infected with *Campylobacter* each year (www.who.int). In several developed countries, the number of reported cases are 80 per 100 000 people (Friedman et al., 2000). In Denmark, 3352 cases (61 pr. 100.000) were reported in 2009 and there has been an increasing trend of campylobacteriosis in Denmark and in most developed countries for the last 25 years (www.ssi.dk; Olson et al., 2008) (figure 1.2). Because *Campylobacter* enteritis rarely requires hospitalization, the number of cases is probably much higher (Friedman et al., 2000). This is supported by data from England and Wales, where it was found that for each reported case there was app. nine others not reported cases (Wheeler et al., 1999). In developing countries, *C. jejuni* is hyperendemic, because of poor sanitation and close human contact with animals. Here, infection is generally asymptomatic and diarrhoea restricted to children less than 5 years (Ketley, 1997). In developed countries, infection is often seasonal and targets mostly young adults (15-24 years) and often men are found to be susceptible (Blaser, 1997; Friedman, 2000).

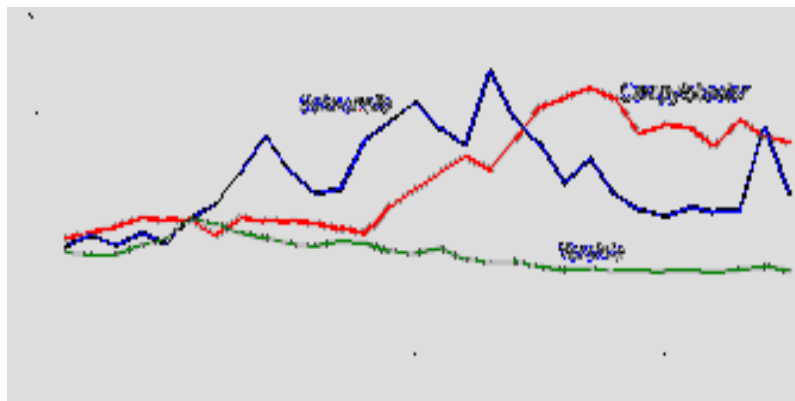


Figure 1.2. Registered *Campylobacter*, *Salmonella* and *Yersinia* patients from 1980 to 2009. The number of registered patients in Denmark have been increasing during the last 25 years topping in 2000-2002.

Campylobacter are found in a wide range of domestic and wild animals and contaminated meat or cross-contaminated food products are usually the routes of infection for humans. However, *Campylobacter* can also be acquired from contaminated and untreated milk, contaminated water, sausages and contact with pets, especially birds and cats, and international travel (Kapperud et al., 1992; Ketley, et al., 1997) (figure

1.3). However, approximately 50% of all infections cannot be attributed to any of the known risk factors, indication that other sources exist (Janssen et al., 2008). Most *Campylobacter* infections occur as sporadic cases, and outbreaks are rare. The few reported outbreaks are most commonly associated with raw milk or water (Engberg et al., 1998; Kuusi et al., 2005; Teunis et al., 2005).

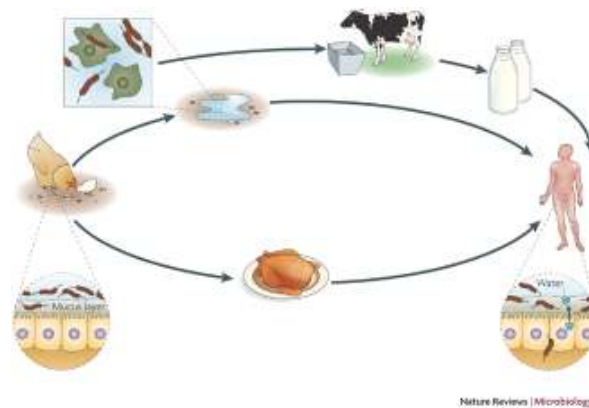


Figure 1.3. The sources of *Campylobacter jejuni* infection. Several sources have been found to contribute to the human infection but especially colonization of chicken is important. During chicken colonization, the *C. jejuni* can spread within the flocks through the faecal-oral route and obtain a high level of contamination within a flock. Thereby humans can be infected by consumption of untreated contaminated poultry products. *C. jejuni* is also able to enter the water supply and thereby survive and infect humans directly through the drinking water or via consumption of animal products, such as meat or milk (Young et al., 2007).

Source tracking is an important issue regarding *C. jejuni* and human campylobacteriosis and good subtyping methods are needed for this. Besides source tracking, some of the typing methods can be very useful to study the bacterial population structure and evolution. Some of the earlier methods are based on phenotypic traits, such as biotyping, serotyping and phagotyping and are still used today. However these methods lack discriminatory power and also results in a large portion of untypeable strains. Since the 1980s there has been an increased interest in the development of molecular typing methods. Several techniques have been developed and have been in general use, such as flagellin gene (*flaA*) sequencing (Ayling et al., 1996), pulse field gel electrophoresis (PFGE) (Fujimoto et al., 1997) ribotyping (Fitzgerald et al., 1996), amplified fragment length polymorphism (AFLP) (Duim et al., 1999) and multilocus enzyme electrophoresis (MLEE) (Selander et al., 1986). *flaA* sequencing analyses restricted parts of the genome, PFGE are solely based on digestion of genomic DNA while AFLP includes two restriction enzymes and a primer pair and makes the method more

discriminatory than *flaA* and PFGE. MLEE analyses variations in alleles of housekeeping genes encoding enzymes by estimating variations in the net electrophoretic charges of the polypeptide. MLEE has a moderate level of discriminatory power but can be difficult to compare between laboratories (Riley, 2004). Overall, none of these methods have proved high resolution, high throughput and simple reliable and easy-to-compare data between laboratories.

In extension of MLEE, multilocus sequence typing (MLST) is another molecular isolate characterization technique. It is based on sequencing parts of the genome and the method indexes variation in seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, *uncA*), which evolve slowly as they are under stabilizing selection for conservation of metabolic function (Dingle et al., 2002). The allele fragments are approximately between 400 and 600 bp in length, which made them easy to sequence on the sequencing instruments available in the mid 1990s, when the method was developed (Maiden, 2006). The use of nucleotide sequence data directly accesses the variation in the targeted gene and the methods are more reproducible and can easily be shared among laboratories. The sharing is easily accessible due to a central database (<http://pubmlst.org/>) where also useful software is available.

The MLST scheme was conceived as a tool for clinical microbiologists and epidemiologists (Urwin and Maiden, 2003) but it has also been proven very useful in the studies of bacterial population genetics (Smith et al., 2000; McCarthy et al., 2007; Sheppard et al., 2008). Studies of *C. jejuni* strain collections, by MLST, have confirmed the organisms to be genetically diverse, with a semi-clonal population structure (Dingle et al., 2001a, 2005). Such bacterial populations contains clusters of related organisms, referred to as clonal complexes and in the case of *Campylobacter*, these are defined as those isolates sharing four or more alleles with a central genotype, after which the complex is named (Dingle et al., 2001a).

1.2 Pathogenesis of *Campylobacter jejuni*

1.2.1 Clinical symptoms and complications

Human infections are caused mainly by two species: *C. jejuni* and *C. coli*.

Manifestations are acute gastroenteritis, cramping abdominal pain, fever, and more rarely, vomiting and headaches (Allos et al., 2001). Diarrhoea often develops shortly after onset of abdominal pain and varies from mild, non-inflammatory, watery symptoms to severe and bloody. In otherwise healthy individuals, infection last for app. 4 days with an incubation period of 1-10 days (Blaser et al., 1997). Disease outcome is most likely to be dependent on virulence of the infecting strain, but also host response and host immune status. This is suggested because patients acquiring infections abroad generally present clinical features of their country of origin (Ketley et al., 1997).

Normally the *C. jejuni* infection is self-limiting but in severe cases, macrolide antibiotic (erythromycin) or fluoroquinolones (ciprofloxacin) is the choice of treatment (Balfour and Faulds, 1993). Resistances to both choices of antibiotic have been increasing among *Campylobacter* species (Engberg et al., 2001a; Gabreel and Taylor, 2006). There is evidence that patients infected with antibiotic-resistant strains have worse clinical outcomes than those infected with sensitive strains (Helms et al., 2005).

Post-infectious complications can occur and Guillain-Barré Syndrome (GBS) is probably the most severe and well studied. It is estimated to be 1 in 1000 infections and causes flaccid paralysis that can affect peripheral and cranial nerves and in severe cases, artificial ventilation is required (Nachamkin, 2002; Kuwabara, 2007). The surface of the bacteria is known to be important for development of GBS because lipooligosaccharides (LOS) mimics the peripheral nerve gangliosides resulting in generation of autoreactive antibodies and thereby inflammation and tissue damage (Godschalk et al., 2004; Komagamine and Yuki, 2006). Miller Fisher syndrome is a non-paralytic variant of GBS and causes inability to move the eyes and having non-reactive pupils (Fisher, 1956). Reactive arthritis is also associated with *Campylobacter* post-infection and a cohort study found a risk of accruing reactive arthritis to be in approximately 7 out of 100 cases (Hannu et al., 2002). There is a maximal interval at 4 weeks between preceding infection and arthritis and the arthritis has a predilection for joints, particularly knees and ankles (Braun et al., 2000). In more rare cases *C. jejuni* has been associated with intestinal haemorrhage (Chamovitz et al., 1983), toxic megacolon (McKinley et al., 1980) and haemolytic uraemic syndrome (Shulman and

Moel, 1983). Also irritable bowel syndrome has been related to *C. jejuni* infection (Gradel et al., 2009).

1.2.2 Virulence factors of *Campylobacter jejuni*

C. jejuni is predicted to express several virulence factors when colonizing the intestines, establishing an infection and possibly getting systemic. Some are more extensively studied than others. Below are highlighted the virulence factors important for host interaction and the following chapters.

1.2.2.1 Motility and flagellin

The motility of *C. jejuni* is important for colonization of the host by the ability of movement into the mucus covering the intestinal cells. *C. jejuni* has a polar flagellum at one or both ends and their cork-screw form allows them to penetrate the mucus barrier (Szymanski et al., 1995). For colonization, the presence of flagellin has been found to be essential (Wassenaar et al., 1993; Nachamkin et al., 1993). Flagellin consists of two subunits encoded by *flaA* and *flaB* and both are subjected to antigenic variation as well as phase variation (Caldwell et al., 1985; Harris et al., 1987). The *flaA* gene is expressed at much higher levels and independently of *flaB*, as *flaA* is expressed from a σ^{28} promoter and *flaB* from a σ^{54} promoter (Guerry et al., 1990). Because *flaA* is expressed at a higher level, the flagellum consists normally of FlaA but both *flaA* and *flaB* have been found to be necessary for motility (Wassenaar et al., 1993). *flaA* has also been associated with autoagglutination (AAG) where a *flaA* mutant failed to autoagglutinate (Guerry et al., 2006). Several other proteins have been found to be involved in flagellar expression and in the annotation of the NCTC 11168 genome, 36 open reading frames were assigned functions or putative functions in flagellar biosynthesis, export or assembly (Parkhill et al., 2000).

1.2.2.2 Adherence factors

When the bacteria have entered the mucus layer, adhesion to the epithelial cell is crucial for long term establishment and invasion. No single mutation completely abolishes binding to host cells, indicating that *C. jejuni* possesses multiple adhesins. Accordingly, several proteins have been proposed as adhesins (table 1.1).

Table 1.1. Overview of known adhesion factors and their importance *in vitro* and *in vivo*.

Adhesin	In vitro cell line	Important for host colonization in	Reference
CadF	Int407	chicken	Monteville et al., 2003; Konkel et al., 1997
FlpA	Int407	chicken	Konkel et al., 2010
CapA	Caco-2	chicken	Ashgar et al., 2007
JlpA	HEp-2	no	Jin et al., 2001, Flanagan et al., 2009
PEB1	HeLa	mouse	Kervella et al., 1993, Pei et al., 1998
PEB4 (surA)	Int407	mouse	Asakura et al., 2007
Cj1496c	Int407	chicken	Kakuda and DiRita, 2006
MOMP	Int407	ND	Moser et al., 1997
CPS	Int407	ferret, chicken	Bacon et al., 2001, Jones et al., 2004
LOS	Int407, Caco2	chicken, mouse	Fry et al., 2000, Müller et al., 2007, Naito et al., 2010

CadF: *Campylobacter* adhesion to fibronectin, FlpA: fibronectin-like protein A, CapA: *Campylobacter* adhesion protein A, JlpA: *jejuni* lipoprotein A, PEB1 and 4: protein Pei, Ellison and Blaser 1 and 4, MOMP: Major outer membrane protein, CPS: Capsular polysaccharide, LOS: Lipooligosaccharide, ND: Not determined

Fibronectin (Fn) is a eukaryotic molecule part of the extracellular matrix (ECM), which serves as binding component for *C. jejuni* (figure 1.4). CadF (*Campylobacter* adhesion to fibronectin) and FlpA (fibronectin-like protein A) are identified as fibronectin-binding proteins, important for adhesion to epithelial cells *in vitro* and chicken colonization (Flanagan et al., 2009).

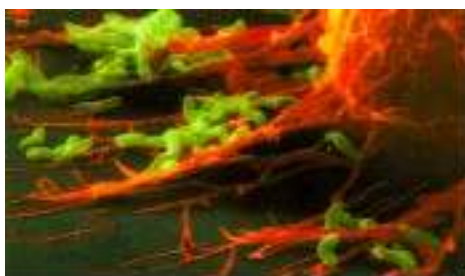


Figure 1.4. *C. jejuni* binding to an extracellular matrix (ECM) component (M. Konkel).

C. jejuni has two putative autotransporters, CapA and CapB (*Campylobacter* adhesion protein A and B), where CapA has been found important for *in vitro* adhesion and chicken colonization (Ashgar et al., 2007). Autotransporters represent, in other pathogens, an extensive and rapidly growing family of secreted virulence-associated proteins (van Putten et al., 2009). JlpA (*jejuni* lipoprotein A) is another characterized

adhesin, crucial for HEp-2 cell binding (Jin et al., 2001). It is a surface exposed lipoprotein that binds to Hsp90- α , localized on the surface of HEp-2 cells. The binding activates NF- κ B and p38 mitogen-activated protein (MAP) kinase, both of which contribute to proinflammatory responses (Jin et al., 2003). The PEB1 and 4 adhesins are paradoxically located in the periplasma but found important for adherence to epithelial cells (Kervella et al., 1993; Pei et al., 1998) and for biofilm formation (PEB4) (Rathbun and Thomson, 2009). Another periplasmic protein, important for adhesion, is the glycoprotein Cj1496c. It has homology to a magnesium transporter and has been shown to be important for adhesion and invasion to human epithelial cells and for chicken colonization (Kakuda and DiRita, 2006). The 46-kDa pore-forming major outer membrane protein (MOMP), encoded by the *porA* gene (Schroder & Moser, 1997) has a hyper variable surface exposure between isolates, probably due to selective pressure by the immune system (Clark et al., 2007). Lipooligosaccharides (LOS) comprises two main components: a hydrophobic lipid A anchor and an oligosaccharide consisting of a conserved inner core and a variable outer core (Golec, 2007). It has previously been shown to be implicated in adherence, invasion and colonization (Fry et al., 2000; Naito et al., 2010). It is highly variable and therefore plays a crucial role in immune avoidance and found to be important for serum resistance (Guerry et al., 2000). Various LOS structures resemble human neuronal gangliosides and this molecular mimicry is thought to lead to the autoimmune disorders, Guillain-Barré and Miller Fisher syndrome (Guerry et al., 2002). The capsule was until recently thought to be a high molecular weight lipopolysaccharide, but is now known to be a highly variable capsular polysaccharide (Karlyshev et al., 2000). The *C. jejuni* capsule is important for adherence and invasion of epithelial cells, chicken colonization and serum resistance (Bacon et al., 2001; Jones et al., 2004; Bachtiar et al., 2007). In addition to LOS and capsule, *C. jejuni* may produce another type of polysaccharide. Its biosynthesis might require carbamylphosphate and it is possibly involved in biofilm formation (Kalmokoff et al., 2006; McLennan et al., 2008). The *C. jejuni* binding to epithelial cells is illustrated at figure 1.5.

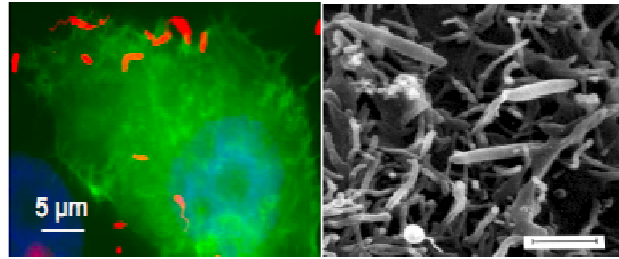


Figure 1.5. *C. jejuni* binding to epithelial cells. Left immunofluorescence image showing *C. jejuni* binding to a INT407 cell and right a scanning electron microscopy image showing *C. jejuni* binding to Caco-2 cells (M. Konkel).

1.2.2.3 Invasion

Early studies of intestinal biopsies and *in vitro* studies of *C. jejuni* showed that the bacterium is able to invade gut tissue cells (van Spreeuwel et al., 1985; Oelschlaeger et al., 1993; Ketley, 1997) and since then, numerous *in vivo* and *in vitro* studies have provided information on the invasion level and mechanisms behind the invasion process. However, still little is known about the mechanisms by which it enters the host cells.

The entrance of the bacteria is mediated through disrupted tight junctions of epithelial cells (Bras et al., 1999; MacCallum et al., 2005; Chen et al., 2006). Subsequently it gets through the epithelial cell barrier either via transcellular or paracellular movements. The latter allows the bacterium to move to the basolateral surface and there either reinvade the epithelial cell or be taken up by macrophages where *C. jejuni* is able to replicate and induce apoptosis (Kielbauch et al., 1985; Hickey et al., 2005). After internalization into intestinal epithelial cells, *C. jejuni* resides within a membrane bound compartment and over time acquires a metabolic state that renders it unculturable under standard culture (Kielbauch et al., 1985; Humphrey et al., 1986; Russel et al., 1994). A study by Watson and Galan (2008) showed that this state could be interrupted by conditions of severe oxygen limitation, suggesting that when *C. jejuni* enters the epithelial cells it either becomes oxygen sensitive or alter its respiration mode (Watson and Galan, 2008). The nucleotide sequence of the genome of the *C. jejuni* 81-176 has further revealed genes involved in additional respiratory pathways and these could contribute to the ability of surviving within epithelial cells (Hofreuter et al., 2006). *C. jejuni* invasion has also been related with the ability to stimulate MAP kinases, leading to the production of the pro-inflammatory cytokine, IL-8 (Hickey et al., 2000; Watson and Galan, 2005).

Upon contact with epithelial cells, *C. jejuni* secretes a set of proteins termed *Campylobacter* invasive proteins (Cia proteins) and at least one of them (CiaB, a 73 kDa protein) has been shown to be crucial for internalization (Konkel and Cieplak Jr, 1992; Konkel et al., 1999). CiaB proteins are also found to be secreted in the presence of chicken serum and mucus (Biswas et al., 2007). The secretion is not mediated by a classical type III secretion system but utilizes instead the flagellum as a type III like secretion system (Konkel et al., 2004).

1.2.2.4 Other factors important for host interaction

A number of other virulence factors and survival mechanisms have been identified in *C. jejuni*, the ones relevant for the following chapters are mentioned below.

Genetic variation and natural transformation

Genetic variation is important to consider while working with or developing new typing methods. *C. jejuni* displays extensive genetic variation caused by intragenomic mechanisms, such as phase variation, gene duplication, deletion, frameshifts and point mutations (Young et al., 2007). Also genetic exchange between strains contributes to the genetic variance and because *C. jejuni* is naturally competent, it can take up DNA from the environment, which leads to recombination between strains and allows the generation of more genetic diversity (de Boer et al., 2002). Genes required for natural transformation have been identified as components of type II secretion system (Avrain et al., 2004). Also a plasmid-encoded type IV secretion system as well as genes for *N*-linked glycosylation, LOS biosynthesis has been shown to be implicated in natural transformation (Bacon et al., 2000; Fry et al., 2000; Larsen et al., 2004).

Biofilm formation and quorum sensing

The formation of biofilm is a well-known bacterial way of growth that increases the ability of bacteria to survive in extreme conditions such as UV radiation, predation and desiccation (Elasri et al., 1999; Matz et al., 2005). The bacteria in biofilms also become more resistant to disinfectants and antimicrobials compared to their planktonic counterparts (Fux et al., 2005). Several studies have shown that *C. jejuni* is capable of

forming biofilm and it is hypothesised to play a role in the dispersal of the bacteria in poultry farms through the water supply (Lehtola et al., 2006). Recent studies have shown that *C. jejuni* biofilm formation is enhanced during aerobic conditions suggesting that the bacteria are well adapted for survival in the environment in a biofilm (Reuter et al., 2010). The molecular details regarding *C. jejuni* biofilm are still deficient but there is evidence that the flagellum is important, because a *flaB* mutant has showed reduced biofilm formation (Reeser et al., 2007) and flagellar modification (Cj1337) mutants do not adhere to glass surfaces (Joshua et al., 2006).

Quorum sensing (QS) is a cell-to-cell communication system that involves the synthesis, secretion and detection of extracellular signalling molecules termed autoinducers. *C. jejuni* do, as most pathogens, produce the signal inducer 2 (AI-2) that is a collection of interchangeable molecules synthesised by a key enzyme, LuxS (Schauder et al., 2001). AI-2 production has been associated with regulation of biofilm formation (Reeser et al., 2007), autoagglutination (Jeon et al., 2003; Guerry et al., 2006), swarming motility (Jeon et al., 2003), transcription of cytolethal distending toxin (CDT) (Jeon et al., 2005), colonization of chickens (Quinones et al., 2009) and sensitivity to hydrogen peroxide (He et al., 2008). AI-2 activity has further been demonstrated in several foods, such as milk, apple juice and chicken broth, suggesting an adaptation to the environment (Cloak et al., 2002).

Cytolethal distending toxin and haemolysin

C. jejuni secretes a toxin termed cytolethal distending toxin (CDT) and is a complex of CdtA, CdtB and CdtC. The three subunits act together to block cell division by performing cell cycle arrest (Lara-Tejero and Galan, 2001). CdtA and CdtC bind to the cell surface helping to deliver the active subunit CdtB, which uses its DNase-I-like activity to cleave dsDNA molecules during the G1 and G2 phase (Whitehouse et al., 1998). CDT is expressed in the bacteria in the colonization of their natural host, chicken, but the chickens do not generate CDT-neutralizing antibodies (AbuOun et al., 2005). The toxin might provide a way to either avoid host immune-response mechanisms or redirect them towards tolerance (Young et al., 2007).

Haemolysis is the breakage of the red blood cell membrane, causing the release of haemoglobin and other internal components. It is a strategy employed by some bacteria to liberate heme for use as iron source (Stintzi et al., 2008). Haemolysins are associated with virulence in several bacterial pathogens and some studies have reported haemolytic activity of *C. jejuni* (and *C. coli*) (Arimi et al., 1990; Misawa et al., 1995). Two genes have been associated with haemolytic activity in *C. jejuni*: *ceuE* and *pldA*. The CeuE protein is a lipoprotein that functions as a periplasmic binding protein. It is a part of an ABC transport system associated with enterobactin transport across the inner membrane (Stintzi et al., 2008). *pldA*, encoding phospholipase A, has been studied for *C. coli* and found to contribute to cell-associated haemolysis (Grant et al., 1997). A *pldA* homologue is present in the genome of *C. jejuni* NCTC 11168 (Parkhill et al., 2000). A putative haemolysin *tlyA* (Cj0588) is also present in NCTC 11168 but the activity has not been proved (Coote et al., 2007).

1.3 Experimental *Campylobacter* infection models

The biological significance of the relatively large number of identified virulence factors still remains unknown. This is mainly because of the lack of suitable infection models. However, the wide diversity of factors that most likely interact to produce disease after infection with *C. jejuni* makes it difficult to find or develop a good animal model. Data suggest that the disease outcome depends on several factors, such as the dose, genetics, innate and adaptive immune status, the enteric microflora and number of repeat challenges (Blaser et al., 1997; Oberhelman et al., 2003; Pazzaglia et al., 1991). A good animal model can be defined as one easy to handle and accessible to the majority of research laboratories, it should be relatively inexpensive and must be reproducible.

Mice provide a convenient model for the study of several pathogenic bacteria, but mice with normal flora and immune system are usually not susceptible to infection with *C. jejuni*. Mice can be transiently colonized, but the model lack robustness for colonization or pathogenesis studies (Young et al., 2000). Mice with a defined limited gut flora have shown promising results for the study of *C. jejuni* pathogenesis. The model leads to reproducible colonization at high levels, resulting in mild inflammation of large intestine followed by clearance after several weeks. SCID mice with limited gut flora,

in contrast, remain colonized at high levels, with tissue inflammation (Chang and Miller, 2006). Another mice model for colonization is a MyD88 mouse deficient in the adapter protein myeloid differentiation factor 88, required for signalling through most TLRs (Watson et al., 2007). Mice deficient for IL-10 have been infected with as little as 10^6 CFU *C. jejuni* NCTC 11168, which produce significant disease and pathological lesions similar to responses seen in humans (Mansfield et al., 2007)

Avian models are reproducible and have also been used widely (Biswas et al., 2006; Hendrixson and DiRita, 2004; Jones et al., 2004; Ringoir et al., 2007) but the bacteria rarely cause disease and therefore is the model more suitable to study colonization and not virulence and therefore limited in terms of understanding the mechanism of enteric infection (Dorrell and Wren, 2007). Other models that have been used are ferrets (Bell and Manning, 1990), hamsters (Humphrey et al., 1985, 1986), dogs (Olson and Sandstedts, 1987), rabbits (Caldwell et al., 1983, Pavlovskis et al., 1991) and pigs (Hu and Kopecko, 2000; Law et al., 2009). The animal model most similar to human infection is the oral infection of non-human primates. *C. jejuni* causes acute enterocolitis in infected macaques and the clinical symptoms resembles the infection observed in humans (Islam et al., 2006; Russel et al., 1989). However this model is rarely used, probably due to economical and ethical reasons.

Progress has also been made in the use of *ex vivo* organ culture model for both human and chicken intestinal biopsies (Grant et al., 2006; Byrne et al., 2007). These assays enable the maintenance of intestinal mucosal tissue samples and the study of both host-pathogen interactions and host-immune responses (MacCallum et al., 2006; Byrne et al., 2007).

1.4 Immune response to *Campylobacter jejuni* infection

Several mechanisms of the innate immune system are involved in the control of *C. jejuni*, including the complement system, and phagocytic activity of macrophages. Humoral immunity also plays an important role in the response against the bacterium.

The innate immune response is a key determinant of subsequent adaptive immune

activation and outcome of infection (Boyd et al., 2007). Innate immunity is initiated by the bacterial contact to the intestinal epithelial cells where host pattern recognition receptors (PRRs), on the surface of intestinal epithelial cells, can respond directly to a pathogen or a pathogen component (pathogen-associated molecular patterns) (Eckmann, 2005). The recognition triggers host signal transduction pathways and the transcription of genes encoding proinflammatory molecules (O'Hara and Shanahan, 2006). The most extensively studied PRRs are the Toll-like receptors (TLRs). The TLR family is a highly conserved group of molecules that play a role in pathogen detection and initiation and regulation of immune responses. Toll-like receptor 5 (TLR5) is found on the apical surface of intestinal epithelial cells. It recognizes conserved epitopes within some bacterial flagellins and initiates signalling that leads to the expression of the gene encoding the chemokine interleukin-8 (IL-8). Studies of *C. jejuni* have demonstrated secretion of IL-8 in response to wild type *C. jejuni* but it is unclear whether TLR5 is involved in the stimulation or if *C. jejuni* has evolved specific adaptations (Hickey et al., 2000; Watson and Galan, 2005). The release of IL-8, and other proinflammatory molecules, activates innate immune defence, such as dendritic cells (DCs) and promotes acquired immune responses (Fleckenstein and Kopecko, 2001) (figure 1.6). DCs play important roles in both the innate and adaptive immune response to microbial pathogens. They are the major antigen-presenting cells and are widely distributed in tissues including the intestinal mucosa (Medzhitov and Janeway, 1997). DCs regulate the type of T-cell mediated immune response to an offending agent and are also a source of proinflammatory cytokines. Further they are the only cells that are able to initiate proliferation in naïve T cells thereby inducing the primary immune response and permitting the establishment of immunological memory (Palucka and Banchereau, 2002). For *C. jejuni* it was recently found that it readily was internalized in DCs over a 2 hr period but after prolonged incubation period (24 hr or 48 hr) only a few viable intracellular bacteria remained (Hu et al., 2006). Further, *C. jejuni* induced the maturation of the DCs as indicated by up-regulation of cell surface marker proteins, stimulation of interleukins and activation of NF- κ B (figure 1.5). When stimulation was made with heat killed *C. jejuni* cells, a minor reduction in cytokine production was observed and a major stimulant was found to be the LOS of the *C. jejuni* cells surface (Hu et al., 2006). Another important effector molecule is the antimicrobial β -defensin. It

is a family of epithelial antimicrobial peptides and also a major component of host innate defence at the gastrointestinal mucosal surface. These have also found to play an important role against *C. jejuni* (Zilbauer et al., 2005)

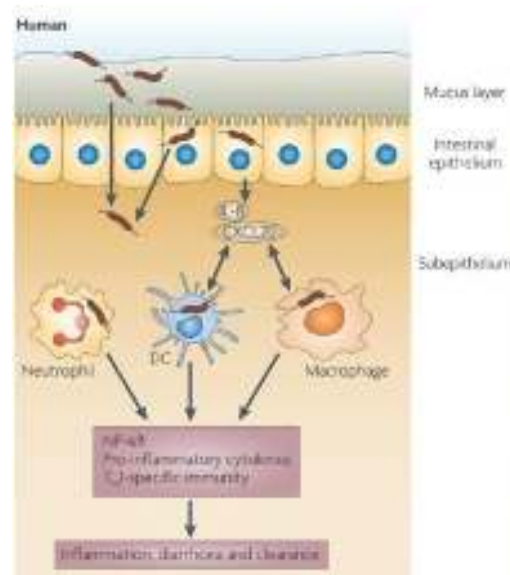


Figure 1.6. Innate immune response to *C. jejuni* infection in humans. *C. jejuni* evade the mucus layer and binds to, and is internalized by, the epithelial cells. This causes interleukin (IL)-8 production that recruits the dendritic cells, macrophages and neutrophils, which interact with *C. jejuni*. These interactions result in a pro-inflammatory response and increase in the corresponding cytokines (Young et al., 2007. Modified).

During the infectious process of *C. jejuni*, most people develop humoral responses to a number of antigens. It has previously been suggested that only a few protein antigens are important in the induction of protective immunity against *Campylobacter* infections (Newell and Nachamkin, 1992). Though, more recent studies have shown that serum antibodies in endemically exposed individuals were directed against a wide range of antigens (Cawthraw et al., 2000). In human, circulation antibodies are first detectable 6 to 7 days after the onset of illness and rise rapidly shortly afterwards (Newell and Nachamkin, 1992). Immunoglobulin (Ig) A levels peak 7 to 10 days after onset of symptoms and decline rapidly after onset of illness. IgG levels peak after 3 to 4 weeks and remain high for a longer time. There is an active secretion mechanism for IgM at mucosal surfaces and IgM levels remains longer than IgA but shorter than IgG (Blaser and Duncan, 1984; Strid et al., 2001; Cawthraw et al., 2002).

1.5 Vaccines against *Campylobacter jejuni*

Until today, no vaccine against *C. jejuni* is available but several attempts have been made so far and still more are coming. Vaccinations against *C. jejuni* are targeted either towards poultry or humans. A vaccine used in poultry production would lead to a reduction or elimination of *C. jejuni* in the animals and thereby reduce the contamination of meat and infections in humans. Vaccination of humans would probably not be of interest for the main population but have a huge importance for immunocompromised patients or travellers.

In *Salmonella typhi* and *Vibrio cholera*, live attenuated oral vaccines, have provided good protection in field or volunteer challenge studies (Black et al., 1990; Kaper et al., 1984) and could be an attractive approach for *C. jejuni* as well. On the other hand, paucity of information on pathogenicity and physiology of *C. jejuni* complicates it. Additionally, a study has showed that viable non-colonizing strain of *C. jejuni* failed to initiate a protective immunity in chickens (Ziprin et al., 2002). Another important subject is the association of the neuropathy and autoimmune disease Guillain-Barré syndrome with *C. jejuni* infections which make the development of whole cell vaccines problematic. Lipo-oligosaccharide from the *C. jejuni* cell wall contains ganglioside-like structures which induces, when injected in rabbits, the antibody cross reaction known to cause neuropathy that resembles acute motor axonal neuropathy (Hughes and Cornblath, 2005).

Killed whole cells used as vaccines have several advantages because they are natural occurring microparticles and could improve interactions between their surface and mucosal lymphoid tissue (Baqar et al., 1995). They are also inexpensive to produce and contain multiple possible protective antigens. A study demonstrated limited protection in ferrets after oral vaccination with *C. jejuni* killed cells (Burr et al., 2005). Subunits or isolated proteins from *C. jejuni* or purified proteins from the bacterial surface are another strategy in vaccine development. Several antigens have been suggested as vaccine candidate. Flagellin is known as immunodominant antigen recognised during infection and therefore has been suggested as vaccine candidate in several studies (Martin et al., 1989; Wenman et al., 1985). Though, the antigenic diversity and the

glycosylation of *Campylobacter* flagellin make the vaccine development problematic (Scott, 1997). The adhesion factor, PEB1, is a highly immunogenic protein, but despite this, a study demonstrates that significant levels of anti-PEB serum IgG did not protect against *C. jejuni* after oral challenge (Sizemore et al., 2005). MOMP has in its native form been found suitable for vaccine design (Zhang et al., 2000; Huang et al., 2007).

Despite more than 10 years of research, the knowledge concerning protective *Campylobacter* antigens is still incomplete. The choice of antigen is a critical point for effective vaccine construction and development of comparative proteomics and/or the analysis of *C. jejuni* metabolic diversity will hopefully facilitate their identification (Jagusztyn-Krynicka et al., 2009).

2. Material and methods

2.1 Overview of material and methods

In figure 2.1 below are the material and methods used in this thesis. They are outlined in details in the following chapters.

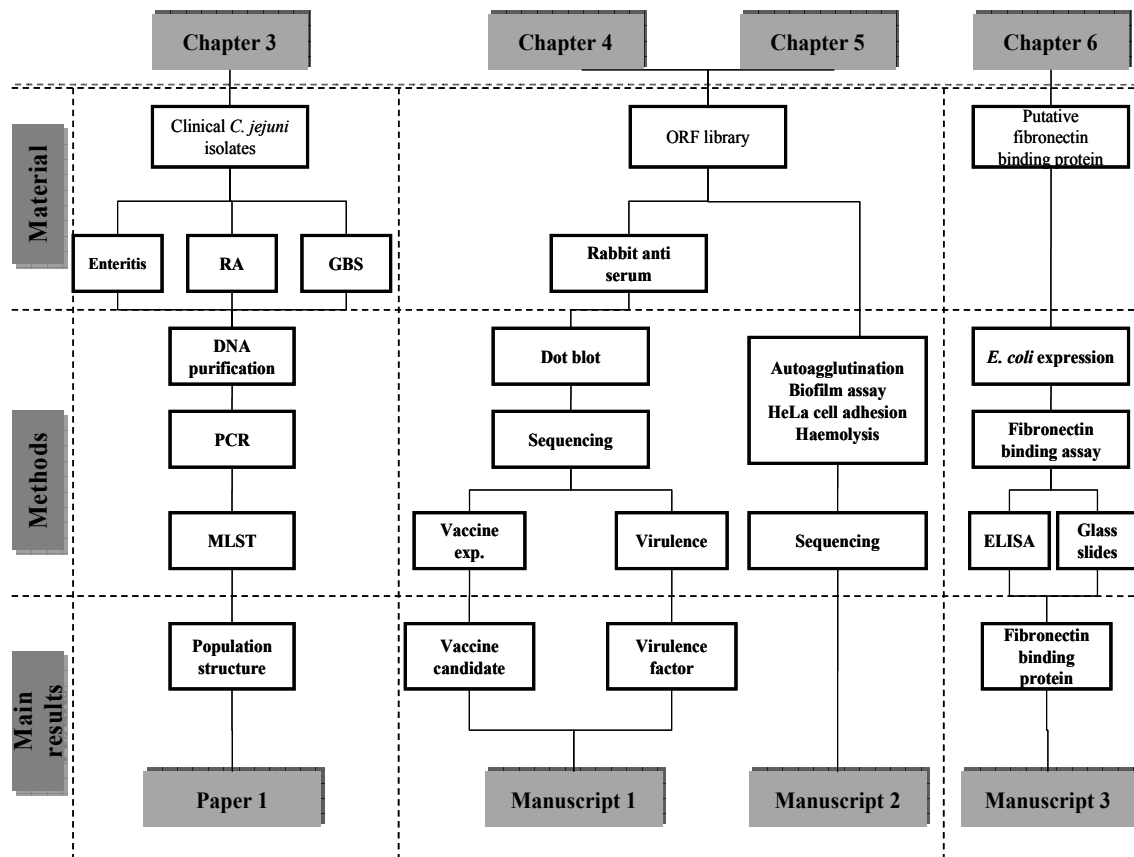


Figure 2.1. Schematic outline of the PhD study. The main material, methods and results are presented in the flow chart. Left and down is a MLST study with clinical isolates. The study is described in details in chapter 3, paper 1 (appendix 2). The two studies outlined in the middle and down both have an open reading frame library as main material with different screen assays. The studies are described in details in chapter 4 and 5, manuscript 1 and 2. The last study, shown at the right and down present a fibronectin binding protein and fibronectin binding assays and is described in details in chapter 6, manuscript 3.

2.1 List of paper and manuscripts

Paper 1 (chapter 3, appendix 2):

MLST clustering of *Campylobacter jejuni* isolates from Patients with Gastroenteritis, Reactive arthritis and Guillain-Barré Syndrome.

Lene N. Nielsen^{1,2}, Samuel S. Sheppard³, Noel D. McCarthy³, Martin C. Maiden³, Hanne Ingmer², Karen A. Krogfelt¹ **1** Statens Serum Institut, Department of Microbial Surveillance and Research, Copenhagen, Denmark. **2** Department for Veterinary Disease Biology, Faculty of life Sciences, University of Copenhagen, Denmark. **3** Department of Zoology, University of Oxford, United Kingdom.

Published in: Journal of Applied Microbiology **108** (2010) **591–599**.

Manuscript 2 (chapter 4):

Identification of immunogenic and virulence associated *Campylobacter jejuni* proteins.

Lene N. Nielsen^{1,2}, Thomas Luijckx³, Christina S. Vegge², Christina Kofoed Johnsen¹, Peit Nuijten³, Karen A. Krogfelt¹, Hanne Ingmer². **1** Statens Serum Institut, Department of Microbiological and Surveillance and Research, Copenhagen, Denmark. **2** University of Copenhagen, Department of Veterinary Disease Biology, Copenhagen, Denmark. **3** Merck, Nobilon International B.V., Boxmeer, The Netherlands.

In preparation

Manuscript 3 (chapter 5):

Screening for virulence associated phenotypes of *Campylobacter jejuni*.

Lene N. Nielsen^{1,2}, Christina S. Vegge², Kirstine A. Frandsen¹, Kaare V. Grunddal¹, Mads P. Jensen¹, Hanne Ingmer², Karen A. Krogfelt¹.

1 Statens Serum Institut, Department of Microbiological and Surveillance and Research, Copenhagen, Denmark, **2** University of Copenhagen, Department of Veterinary Disease Biology, Copenhagen.

Ongoing work

Manuscript 4 (chapter 6):

Identification of a fibronectin binding protein of *Campylobacter jejuni*

Lene N. Nielsen^{1,2}, Christina S. Vegge², Karen A. Krogfelt¹, Hanne Ingmer², Michael Konkel³

1 Statens Serum Institut, Department of Microbiological and Surveillance and Research, Copenhagen, Denmark, **2** University of Copenhagen, Department of Veterinary Disease Biology, Copenhagen, Denmark, **3** Washington State University, School of Molecular Biosciences, Pullman, Washington, United States of America.

In preparation

3. MLST clustering of *Campylobacter jejuni* isolates from patients with gastroenteritis, reactive arthritis and Guillain-Barré Syndrome

3.1 Abstract

Aims: To determine the diversity and population structure of *Campylobacter jejuni* (*C. jejuni*) isolates from Danish patients and examine the association between MLST types and different clinical symptoms including gastroenteritis (GI), Guillain-Barré Syndrome (GBS) and reactive arthritis (RA). **Methods and Results:** Multilocus sequence typing (MLST) was used to characterize 122 isolates, including 18 from patients with RA and 8 from patients with GBS. The GI and RA isolates were collected in Denmark in 2002-2003 and the GBS isolates were obtained from other countries. In overall, 51 sequence types (STs) were identified within 18 clonal complexes (CCs). Of these three CCs, ST-21, ST-45 and ST-22 clonal complexes accounted for 64 percent of all isolates. The GBS isolates in this study significantly grouped into the ST-22 clonal complex, consistent with the PubMLST database isolates. There was no significant clustering of the RA isolates.

Conclusions: Isolates from Denmark were found to be highly genetic diverse. GBS isolates grouped significantly with clonal complex ST-22 but the absence of clustering of RA isolates indicated that the phylogenetic background for this sequela could not be reconstructed using variation in MLST loci. Possibly, putative RA-associated genes may vary, by recombination or expression differences, independent of MLST loci. **Significance and Impact of the Study:** MLST typing of *C. jejuni* isolates from Danish patients with gastroenteritis confirmed that the diversity of clones in Denmark is comparable to that in other European countries. Furthermore, a verification of the grouping of GBS isolates compared to RA isolates provides information about evolution of the bacterial population resulting in this important sequela.

3.2 Introduction

Campylobacter jejuni is the leading bacterial cause of gastroenteritis in the industrialized world causing almost 500 million annual cases worldwide (Friedman et al., 2000). In Denmark, the prevalence of campylobacteriosis was 71 cases per 100.000 in 2007 (www.ssi.dk). The number of laboratory-confirmed cases has increased in

Denmark over a 10-year period from 2665 cases in 1997 to 3868 cases in 2007 and currently accounts for more than twice of notifications of *Salmonella*, Consistent with other developed countries. Most *Campylobacter* infections are sporadic and several risk factors have been identified including consumption of raw milk or contaminated water, red meat and poultry, contact with pets (especially birds and cats), and international travel (Kapperud et al., 1992).

The symptoms of campylobacteriosis vary from diarrhoea to severe invasive disease and sequelae including Guillain-Barré syndrome (GBS), a demyelinating disorder resulting in acute muscular paralysis. Affected people develop weakness of the limbs and the respiratory muscles and areflexia (Nachamkin et al., 1998). Approximately one case of GBS occurs for every 1,000 cases of campylobacteriosis and of these, 20 % are left with some disablement, sometimes needing mechanical ventilation. Approximately 2 to 3 % of cases result in death with many more occurring in the developing world (Willison and O'Hanlon, 2000). GBS is believed to be a result of molecular mimicry of lipooligosaccharide, a part of *C. jejuni* cell envelope, and sugar moieties on nerve gangliosides. Antibodies raised during *Campylobacter* infection containing such ganglioside mimics, can cross-react with gangliosides in the patients and lead to demyelination of nerves and degeneration of axons (Nachamkin et al., 1999; Ang et al., 2004).

Campylobacteriosis is also associated with another immune-mediated sequela, reactive arthritis (RA), a reactive arthropathy. It occurs in between 0.6% and 24% of the patients (Pope et al., 2007). Multiple joints can be affected, in particular the knee joint, with symptoms of pain and incapacitation usually resolving completely within several months (Jansen et al., 2002).

Discriminatory typing methods to study the population genetics of *C. jejuni* isolates are crucial to improve our understanding of the epidemiology and genetic background of this pathogen. Multilocus sequence typing (MLST) has proved to be a valuable typing tool for discriminating *Campylobacter* isolates and defining population structure (Dingle et al., 2001a). MLST is based upon an allelic profile obtained by sequence

analysis of seven housekeeping genes. The allelic profile is summarized by a sequence type assigned using an online database (PubMLST). Relatedness can be inferred and isolates can be grouped as clonal complexes. The advantages of MLST, compared to other molecular methods, such as pulse-field gel electrophoresis, are standardized nomenclature, free access to the database and direct comparability of results between different studies/laboratories.

Previous studies have shown that certain genotypes are more common cause of disease in humans while others may be less important (Manning *et al.* 2003; Siemer *et al.*, 2004, Sheppard *et al.*, 2009a and b). In this study, the genetic diversity among isolates from human disease in Denmark was investigated and the relationship between Sequence Types (STs) and Clonal Complexes (CCs) and different clinical symptoms of the patients was determined.

3.3 Material and Methods

3.3.1 Strain collection

During the period 2002-2003 consecutive faecal isolates were obtained in the diagnostic laboratory at SSI. On a follow up study for sequelae cases of human gastroenteritis (n = 96), reactive arthritis (n = 18) and GBS (n=0) were defined (Schiellerup *et al.* 2008). Since no GBS cases were detected during the investigation, 8 previously described GBS isolates originated from China, Japan and Mexico were included in the study (Engberg *et al.*, 2001; Nachamkin *et al.*, 2002; Leonard *et al.*, 2004). In the phylogeny analysis, additional GBS MLST types were further extracted from the MLST database (PubMLST).

3.3.2 Bacterial growth and preparation of chromosomal DNA

The isolates were cultured on *Campylobacter* blood free medium (mCCDA) agar plates (SSI, LAB112) and subsequently grown on blood agar plates with 5 % yeast for 24-48 hours at 37°C under microaerophilic conditions. For isolation of chromosomal DNA, a suspension of *C. jejuni* cells was prepared in 250 µl PBS in a 0.5 ml eppendorf tube. The suspension was vortexed briefly, heated at 100°C for 10 min and centrifuged at 13,000 rpm for 10 min. The supernatant was removed and stored at -20°C until it was

required for PCR amplification.

3.3.3 PCR amplification and sequencing

Internal fragments of seven gene targets (*aspA*; *glnA*; *gltA*; *glyA*; *pgm*; *tkt*; *uncA*) were amplified by PCR with primers stated at the MLST database (PubMLST). The amplification reaction mixture comprised ~ 10 ng campylobacter chromosomal DNA, 1 µM each PCR primer, 1X PCR buffer, 1.5 mM MgCl₂, 0.8 mM deoxynucleoside triphosphates and 1.25 U of Amplitaq polymerase. Reaction conditions: 95°C for 3 min, 35 cycles of 94°C for 20 sec, annealing temperature for each primer set at 50°C for 20 sec, and extension at 72°C for 1 min, with a final extension step for 5 min. PCR product was confirmed by agarose gel electrophoresis. PCR products were purified by precipitation with 20% polyethylene glycol-2.5 M NaCl and their nucleotide sequences were determined on each strand with BigDye Reaction mix in accordance with the manufacturers' instructions.

3.3.4 Allele and ST assignment

Sequences were commercially determined on both DNA strands and assembled from resultant chromatograms using the Staden suite of computer programs (Staden, 1996). Consensus sequences for each allele were assigned an allele number and the 7-locus (3309bp) ST by interrogation of the *Campylobacter* MLST database (<http://pubmlst.org/campylobacter/>). Novel alleles and STs were submitted to the MLST database to obtain new numbers.

3.3.5 Phylogeny analysis

7-locus allelic profiles were concatenated and used to construct genealogies using two methods for inferring evolutionary relationships among *C. jejuni* STs. First relatedness of isolates was represented by a dendrogram constructed by cluster analysis using the unweighted pair group method with arithmetic mean (UPGMA) in the program START2, available at www.pubmlst.org (Jolley et al., 2001). The second phylogenetic analysis estimated the clonal genealogy of STs using the model-based approach to determining bacterial microevolution: CLONALFRAME (Didelot and Falush, 2007). This is a model that calculates clonal relationships with improved accuracy as it distinguishes

point mutations from imported chromosomal recombination events – the source of the majority of allelic polymorphisms. Analysis was carried out on concatenated sequences representing 51 STs, from 122 isolates from RA, GBS, and GI. The program was run with 50,000 burnin followed by 50,000 subsequent iterations. The consensus trees represent combined data from three independent runs with 50% majority rule consensus required for inference of relatedness.

Because of statistical limitations, we included and compared the typed GBS isolates with the GBS isolates in the PubMLST database which are distributed with the complexes ST-22 (30.5%), ST-21 (16.5%), ST-403 (8%), ST-508, 61 and 42 (5.5%), ST-48, 658, 52, 362, 607 and 206 (2.7%) and isolates currently unassigned to a lineage with 11% (<http://www.mlst.net>).

3.3.6 Statistical analysis

Association between the clonal complex and the clinical diagnosis was assessed by Fisher's exact test (also known as the Fisher-Freeman-Halton test) using software SAS (Sas Institute, 2008) Counts from the rare CCs were grouped into category Other.

3.4 Results

3.4.1 Diversity of sequence types (ST) and clonal complexes (CC)

Among the 122 isolates, 51 STs clustering in 18 clonal complexes were identified. Three clonal complexes; ST-21, ST-45 and ST-22, predominated and accounted for nearly 64% (78/122). In the ST-21 complex, ST-388 and ST-53 were the most common sequence types each representing 6.6%. The ST-45 complex, the second most common group, was dominated by ST-45 with 13.9%. The ST-22 complex represented only two groups, ST-22 and ST-567, with the ST-22 as main sequence type with 9.9%. Despite the fact that the ST-21 complex was the most frequent group, the subgroup ST-45 in ST-45 complex was found to be the most common, accounting for 17 out of 122 isolates. A number of isolates were found in CCs only represented by one or two STs (table 3.1).

Table 3.1. Sequence types and clonal complexes among 122 human isolates grouped as clinical disease and results in parenthesis from Fishers exact test ($p=0.0022$) for the most frequent CCs (21, 22, 42 and 48). For CCs with more than one isolate, the accumulated value is shown in the row for the last isolate.

Isolate number	asp	gln	glt	gly	pgm	tkl	unc	ST ^a	CC ^b	diag	ST freq. (FE)
1575, 1576, 1580, 1581	1	3	6	4	3	3	3	22	22	GBS	4
1577	1	3	6	4	3	3	1	567	22	GBS	1 (0.918)
1579, 1582	1	2	42	4	256	9	8	1672	42	GBS	2 (0.33)
1578	8	10	16	2	11	12	6	2049	354	GBS	1 (1.90)
1319	7	1	52	83	2	3	6	3708	UN ^c	RA	1
1372	33	160	30	79	19	62	5	3614	UN	RA	1
1209	33	160	30	79	104	62	5	UN	UN	RA	1
1152, 1336	2	1	1	3	2	1	5	21	21	RA	2
1142, 1149, 1172	2	1	21	3	2	1	5	53	21	RA	3 (5.61)
1015, 1313	1	3	6	4	3	3	3	22	22	RA	2 (2.06)
1092, 1231	4	7	10	4	1	7	1	45	45	RA	2
1037	4	7	10	4	42	7	1	137	45	RA	1
1014, 1046	4	7	10	10	2	3	1	2589	45	RA	2 (4.13)
1093, 1094	2	4	1	2	19	62	5	2955	48	RA	2 (1.18)
1312	1	4	2	2	6	3	1	219	61	RA	1
1515	2	1	12	121	2	1	5	3709	UN	GI	1
1417, 1418, 1566	2	1	1	3	2	1	5	21	21	GI	3
1535, 1551	2	1	12	6	2	1	5	24	21	GI	2
1540, 1542	8	1	6	3	2	1	1	44	21	GI	2
1032, 1525, 1567, 1571	2	1	12	3	2	1	5	50	21	GI	4
1526, 1543, 1544, 1545, 1548	2	1	21	3	2	1	5	53	21	GI	5
1058, 1530, 1550	2	1	5	2	2	1	5	251	21	GI	3
1025, 1076, 1416, 1422*	43	1	1	3	2	1	5	388	21	GI	8
1517	2	1	5	3	3	1	5	815	21	GI	1
1506	2	7	12	3	2	1	5	1461	21	GI	1
1520	2	1	1	4	1	62	5	1820	21	GI	1
1516	2	1	177	3	2	1	5	1823	21	GI	1
1565	2	1	4	3	2	1	5	2057	21	GI	1 (29.9)
1047, 1049, 1084, 1528, 1564, 1574	1	3	6	4	3	3	3	22	22	GI	6
1519	1	2	42	4	3	3	3	2615	22	GI	1 (11.02)
1023, 1533, 1539	1	2	3	4	5	9	3	42	42	GI	3 (3.93)
1006, 1091, 1507, 1508, 1510**	4	7	10	4	1	7	1	45	45	GI	15
1028, 1531, 1532	4	7	10	4	42	7	1	137	45	GI	4
1509, 1511	4	7	10	4	42	51	1	583	45	GI	2
1504	4	2	10	4	1	7	1	782	45	GI	1
1549	4	7	10	4	1	7	4	2406	45	GI	1 (22.03)
1057, 1537	2	4	1	2	7	1	5	48	48	GI	2
1524	7	4	5	2	11	1	5	429	48	GI	1
1500	2	4	1	4	19	62	5	475	48	GI	1
1033, 1043	43	4	1	2	7	1	5	505	48	GI	2 (6.29)
1523	3	1	5	17	11	11	6	49	49	GI	1
1569	9	25	2	10	22	3	6	52	52	GI	1
1534	2	21	5	3	23	1	5	290	206	GI	1
1027, 1522, 1554, 1562	9	2	4	62	4	5	6	257	257	GI	4
1513	9	17	5	62	4	5	6	2543	257	GI	1
1074	7	1	2	2	4	3	6	82	353	GI	1
1538	7	17	5	2	10	3	6	353	353	GI	1
1512	9	112	5	2	11	3	6	936	353	GI	1
1527	7	2	5	2	2	3	6	2132	353	GI	1
1071	7	17	5	2	13	3	6	3510	353	GI	1
1552, 1568	8	10	2	2	11	12	6	354	354	GI	2
1502, 1536	10	27	43	19	6	18	7	270	403	GI	2
1570	7	17	2	15	23	3	12	51	443	GI	1
1518	7	17	16	15	23	3	12	2687	443	GI	1
1298	1	6	60	24	12	28	1	508	508	GI	1
1501	10	1	50	99	120	76	52	678	677	GI	1
1503, 1561	10	81	50	87	120	76	52	794	677	GI	2
1573	2	1	29	28	58	25	58	1332	1332	GI	1

* + 1553, 1557, 1563, 1572

** + 1514, 1521, 1529, 1541, 1547, 1555, 1556, 1558, 1559, 1560

^a Sequence type

^b Clonal complex

^c UN, currently unassigned to a lineage

3.4.2 Association between clonal complexes and clinical symptoms.

The distribution of the 122 *C. jejuni* isolates and comparison of the gastroenteritis, GBS and RA isolates were investigated (figure 3.1). The isolates from gastroenteritis were represented in all CCs except ST-61. GBS isolates were found in ST-22, 42 and 354 complex. Isolates from RA were found in the ST-21, 45, 42, 48 and 61 complexes. The isolates from gastroenteritis were most frequently represented with the ST-21 (34%) and ST-45 complexes (23%), GBS isolates with the ST-22 (63%) and ST-42 complexes and RA more evenly distributed with the complexes ST-21 (28%), ST-45 (28%), ST-22 (11%) and ST-48 (11%).

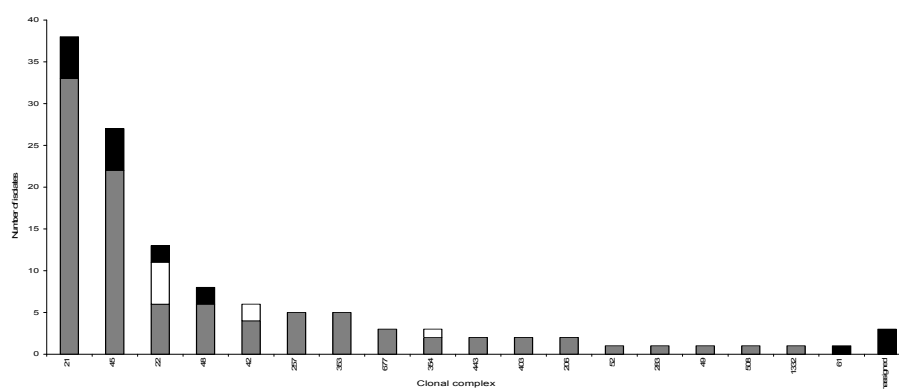


Figure 3.1. Distribution of the CCs among the 122 isolates with different clinical outcomes in this study. GI are shown in grey, GBS in white and RA in black. The ST-21 complex is shown to be the most frequent clonal complex followed by the ST-45 and ST-22 complexes. The GBS isolates are represented in the ST-22, ST-42 and ST-354 complexes and found to be significantly overrepresented in the ST-22 complex.

The clonal complex frequencies varied within the three clinical outcome groups. For most frequent CCs appearing in at least two different clinical diagnosis types, the significance of association with diagnosis was confirmed by Fishers exact test. Both the observed frequency and the frequency expected under the hypothesis of no association (homogeneity) are given (Table 3.1). The cell frequencies show far more observed ST-22 and ST-42 complexes than expected among GBS patients. Fishers exact test demonstrates a significant association between the CC and the diagnosis ($p=0.0022$).

3.4.3 Phylogeny and clustering of the isolates

The UPGMA dendrogram cluster analysis (figure 3.2) showed a grouping of isolates in relation to clonal complexes (shown with brackets). The sequelae (GBS and RA), marked in bold, were not found to clearly cluster within these groups. There is,

however, some evidence of clustering of GBS strains within the ST-22 complex even though there is a limited number of isolates. On the clonal frame genealogy (figure 3.3), the three different symptoms are found to be distributed evenly in the tree, not indicating any clustering of CCs or STs according to symptoms.

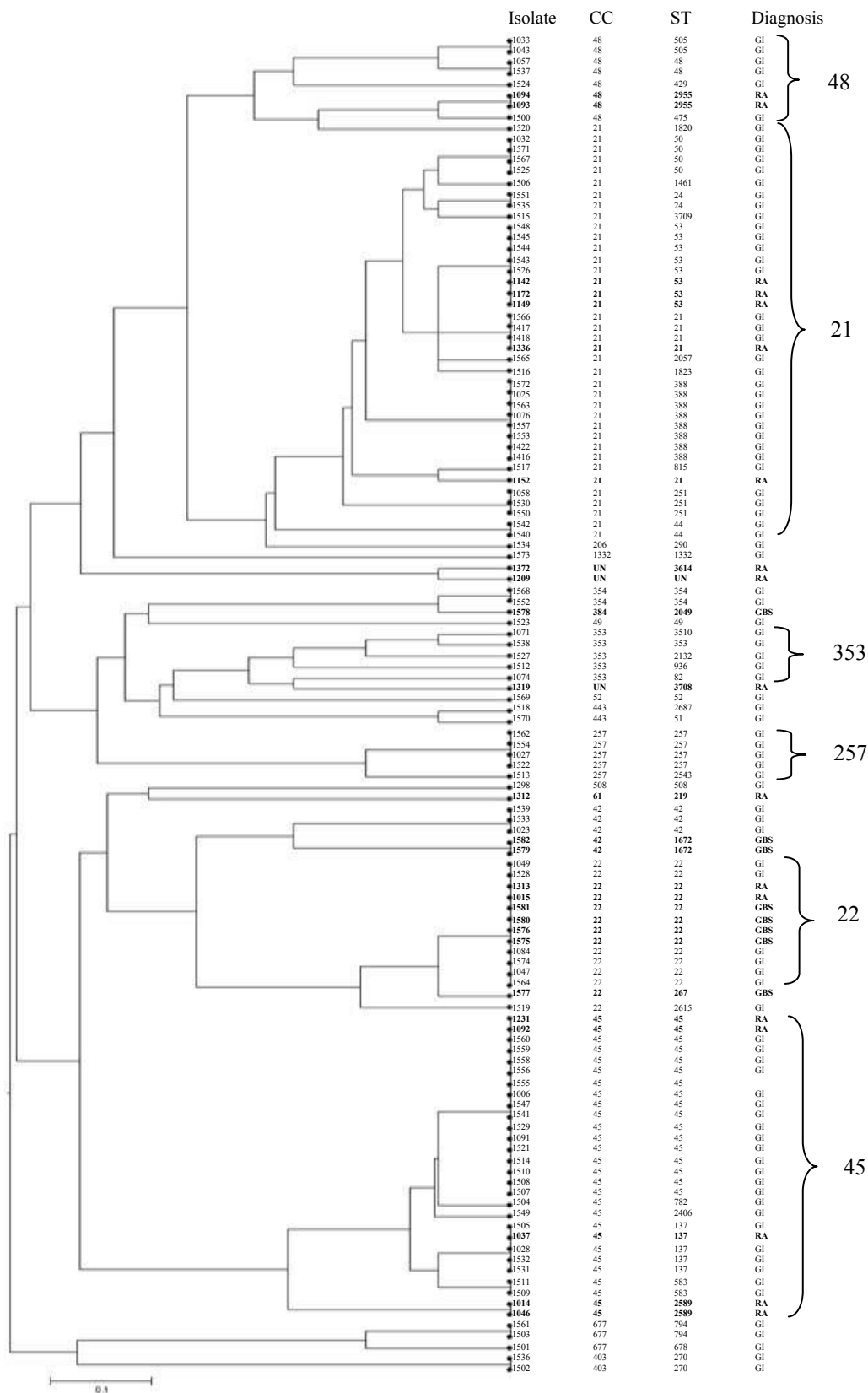


Figure 3.2. Dendrogram demonstrating the phylogenetic relationship between the 122 isolates. Majority of strains were from patients with GI, strains from patients with sequelae (RA and GBS) are marked in bold. The isolates clustered in relation to clonal complexes (marked with brackets). A minor clustering of GBS to ST-22 was observed.

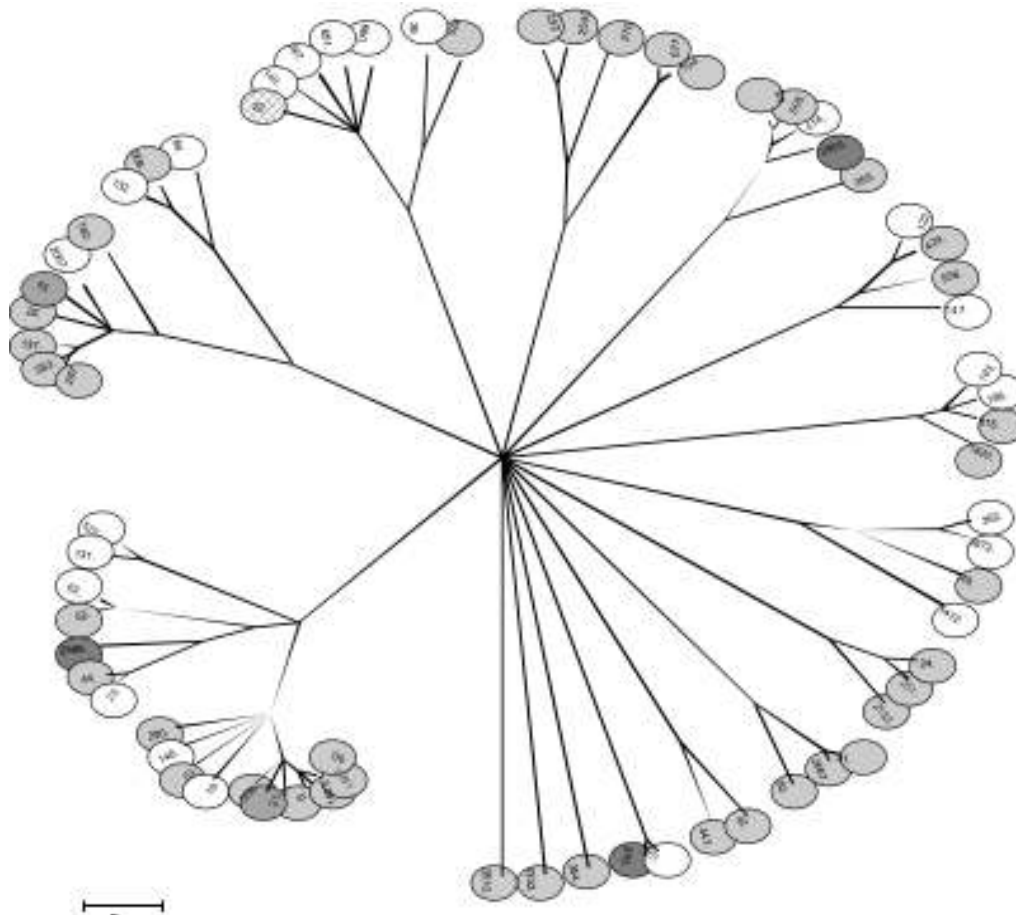


Figure 3.3. Clonal frame tree demonstrating the genetic relationships of STs between *C. jejuni* isolated from patients with GI, RA and GBS. GI are shown as light grey, GBS white and RA dark grey. Combinations of sequelae are shown as GI + RA + GBS with squares and GI + RA with lines. The clonal frame tree includes GBS isolates from the MLST database (PubMLST).

3.5 Discussion

We have examined the association between *C. jejuni* genotypes and different clinical sequelae. *C. jejuni* isolates from a range of sources, geographical locations in Denmark and patients diagnosed with either gastroenteritis or RA were discriminated and formed the basis of our study. GBS isolates were added to the dataset from previously described cases outside the studied population. By using MLST the Danish isolates proved to be highly diverse with a total of 51 sequence types belonging to 18 clonal complexes. This finding is consistent with other studies examining *C. jejuni* isolates from a single geographic location (Duim et al., 2003; Mickan et al., 2007). However, our MLST data also identified a number of frequently described sequence types that have formerly been associated with human infection (Manning et al., 2003; Dingle et al., 2005; Sheppard et al., 2009b).

The ST-21 complex was particularly common with 38 %, similar to previous studies, where it accounted for up to 20 to 33 % of the isolates (Dingle et al., 2001a; Schouls et al., 2003; Sopwith et al., 2006, Karenlampi et al., 2007). The prevalence of the ST-21 complex in Danish isolates is also found to be consistent with the MLST surveys of *Campylobacter* in other European countries, (Duim et al., 2003; Mickan et al., 2007; Kwan et al., 2008; McTavish et al., 2008). The other major clonal complex in our study, the ST-45 complex, accounted for approximately one quarter of the isolates and the ST-48 complex was found with the frequency of 6.6%. These complexes have also been identified in other countries and from multiple sources (Dingle et al., 2001a; McTavish et al., 2008). Isolates from patients diagnosed with RA were not found to be associated with one or few of the clonal complexes. Therefore, we suggest that specific RA features rather involve differential expression of virulence factors that might be revealed by expression analysis of RA isolates or because of rapid recombination of disease associated genes. Furthermore, host specific genetics might be involved.

The ST-22 complex accounted for 10% of the isolates and interestingly it was significantly overrepresented in the collection of GBS isolates both in our study as well as in the PubMLST database. The ST-22 complex was also described in isolates from several countries and animal sources (Duim et al., 2003; Kwan et al., 2008) but not as frequently as the ST-21 and ST-45 complexes. A study by Dingle et al. 2001b suggested a possible relatedness between the ST-22 complex and GBS isolates and this notion is supported by our data. Furthermore, the authors found that the ST-45 complex was underrepresented among GBS isolates (Dingle et al., 2001b). Once more our data and the GBS collection in the MLST database confirm this by the fact that to date no GBS isolates carry the ST-45, despite the fact that this is the most common sequence type identified in our study. The association between GBS and the two clonal complexes ST-22 and ST-42 could be explained by a more frequently expression of the GBS-related Gm1 gangliosides, but we have no evidence of this. By comparison of the Danish isolates with the PubMLST database (including the global isolates), we found that the Danish isolates are similar to those obtained from other parts of the world and therefore, geographical location of the isolates is not correlated with sequence type. In addition, the global GBS isolates have been compared with the GBS isolates in our study and

shows the same results. In an earlier study, the GBS isolates were analysed by other methods and the population was found to be heterogenic (Engberg *et al.* 2001). Further analysis of the GBS isolates by DNA microarrays confirmed significant genomic heterogeneity among the isolates (Leonard *et al.*, 2004). Despite these results, we believe that the results presented here together with those of others (Dingle *et al.*, 2001b) suggest a possible correlation between certain complexes, such as ST-22 complex, and the development of GBS.

3.6 Acknowledgments

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4. Identification of immunogenic and virulence associated *Campylobacter jejuni* proteins

4.1 Abstract

With the aim of identifying proteins important for host interaction and virulence, we have screened an expression library of NCTC 11168 *C. jejuni* genes for highly immunogenic proteins. A commercial *C. jejuni* ORF library consisting of more than 1600 genes was transformed into the *E. coli* expression strain BL21 (DE3) resulting in 2304 clones. This library was subsequently screened for immunogenic proteins using antibodies raised in rabbit against a clinical isolate of *C. jejuni*; this resulted in 52 highly reactive clones, which represented 25 different genes after sequencing. Selected candidate genes were inactivated in *C. jejuni* NCTC 11168 and the virulence was examined using INT 407 epithelial cell line. These investigations revealed Cj0034 to be a novel virulence factor and support the usefulness of the method.

4.2 Introduction

The food-borne pathogen, *Campylobacter jejuni* is a gram-negative, microaerophilic, spiral-shaped and motile bacterium. It is the most common cause of food- and water-born gastroenteritis worldwide causing approximately 500 million human infections every year (Friedman et al., 2000; Skirrow et al., 2000). Infection is often associated with consumption of undercooked poultry meat, but water and other food sources also play a great role in the transmission of *C. jejuni* (Friedman et al., 2000). The symptoms of campylobacteriosis range from mild non-inflammatory, watery, self-limiting diarrhoea to severe abdominal cramps and bloody diarrhoea with fever and vomiting. Also post-infectious complications as reactive arthritis and Guillain-Barré syndrome are found to be associated with *C. jejuni*.

Bacterial surface structures can interact with host tissue and could be responsible for the ability of *C. jejuni* to colonize the gastrointestinal tract of humans, which is believed to be essential for infection. A study has shown that *C. jejuni* isolated from patients with fever and diarrhoea revealed greater binding to epithelial cells compared to isolates from patients without fever and diarrhoea (Fauchere et al., 1986). Several mechanisms

involved in the survival and persistence of the bacteria in the gut are known, but far from enough to understand the complexity of virulence. Colonization of the gut is promoted by flagellar-mediated motility (Carrillo et al., 2004) and binding to host tissue like e.g. to fibronectin mediated by CadF and 1279c (Konkel et al., 1997). Furthermore, several other outer membrane proteins are found to be implicated in colonisation including MOMP (Moser et al., 1997), PEP1 (Leon-Kempis et al., 2006), Omp50 (Bolla et al., 2000), lipoproteins Omp18 (Burnens et al., 1995, Konkel et al., 1996), JlpA (Jin et al., 2001) and Cia proteins (Rivera-Amill et al., 2001). In addition, some of these surface exposed proteins are found to be immunogenic (Pei et al., 1998, Burnens et al., 1995), which opens the possibility of vaccine development. Humoral immune response to a number of *C. jejuni* antigens is developed in most people upon an infection and epidemiological studies indicate that the immunity is crucial for the development of protection against *Campylobacter* disease (Tribble et al., 2010).

The purpose of this study was to identify novel *C. jejuni* antigens and potential new virulence factors by screening a *C. jejuni* ORF expression library (Parrish et al. 2004) with serum from immunised rabbits. Selected candidates of the identified genes were examined for their role in virulence and tested as potential vaccine candidates.

4.3 Material and methods

4.3.1 Bacterial strains and plasmid

The bacterial strains used in this study included *E. coli* SURE (Stratagene) and *E. coli* BL21 (DE3) (Stratagene) and the plasmid was pTLJ03. Strains and plasmid originates from a NCTC 11168 *C. jejuni* ORF library (Parrish et al., 2004) available from Geneservice. The expression clone set comprises of >1,600 *C. jejuni* ORF's and the expression vector pTLJ03 generates N-terminal GST-His-tagged fusion proteins. Strains were grown in LB media or the expression media MagicMedia (Invitrogen) at 37 °C. pTLJ03 containing strains were grown in media containing 50 µg/mL ampicillin unless otherwise specified. *C. jejuni* strains (NCTC 11168 and NCTC 11168H) were grown at 37 °C microaerophilic on blood plates (BaseII and 5 % blood) in BHI broth or biphasic (blood plates and BHI broth) with antibiotic when needed (30 µg/mL kanamycin or/and 50 µg/mL streptomycin).

4.3.2 Expression library

The library was originally created in *E. coli* SURE for optimal storage. The strain does not contain the T7 polymerase and for that reason the library was transformed to the *E. coli* BL21(DE3) expression strain. The clones were grown separately in microtiter plates in 200 µl LB media containing ampicillin overnight and subsequently the plasmids were purified as a pool and transformed to the chemocompetent *E. coli* BL21 (DE3) strain. This revealed an expression library consisting of 2304 clones (24 microtiter plates).

4.3.3 Immunoblot assay

Individual clones were grown 16-20 hrs in microtiter plates in MagicMedia for optimal expression. 2 µl of the culture was spotted on nitrocellulose membranes. The membranes were blocked in blocking buffer 30 min., washed in PBS tween and then incubated in primary antibody (1:1000) at 4°C for 16-20 hrs. The membranes were then washed in PBS tween and incubated in secondary antibody (Polyclonal goat anti rabbit immunoglobulins/HRP, Dako) for 1 hr. The reaction was visualised by chemoluminescence (chemoluminescent substrate, Invitrogen). The primary antibody was raised in rabbit immunised with a boiled-treated (100°C for 1 h) *C. jejuni* Penner serotype 2 originally isolated from a human patient. The Penner serotype 2 serum was chosen to correspond the serotype used in the commercial library (NCTC 11168). The serum was preincubated with *E. coli* BL21(DE3) before use to minimise background reaction. To verify that the antigens also reacted against human serum, a dot blot with 10 selected clones expressing antigens and serum isolated from a patient infected with *C. jejuni* Penner serotype 2 was carried out as described above.

4.3.4 Clone sequencing

Plasmid DNA was isolated from 100 ml *E. coli* BL21(DE3) cultures using MidiPrep (Qiagen). Sequencing was conducted by Macrogen Inc. and the primer 5'GCT ATC CCA CAA ATT GAT AA 3'.

4.3.5 Recombinant DNA techniques

Knock out mutants were kindly provided by Brendan Wren from the London School of Hygiene and Tropical Medicine, University of London. Their constructs were made via insertion of the Km cassette into unique sites present in pUC18-based recombinant plasmids containing random 1-2 kb fragments of the *C. jejuni* NCTC 11168 genome.

Subsequently the gene knock outs were transferred from the 11168H strain used in Brendan Wrens laboratory to the 11168 strain used in our lab. Natural transformation was performed as described previously (Wang and Taylor, 1990) with some modifications. *C. jejuni* cultures grown overnight on BHI agar plates were collected and resuspended in 12 ml BHI broth to OD₆₀₀ of 0.001. Bacterial suspensions in three dilutions were transferred to sterilized Petri dishes, incubated at 37°C with no shaking under micro aerobic conditions over night. 200 µg cultures with OD₆₀₀ 0.2-0.3 were transferred to sterilized tube with 1 ml BHI and incubated at 37°C with shaking under micro aerobic conditions 2 h. Then 10 ng of genomic DNA, purified with Qiagen blood and tissue kit) of the mutants, was added each tube. After additional incubation for 3 h, bacterial cultures were serially diluted and plated on BaseII agar plates with antibiotics (50 mg/l kanamycin). The agar plates were incubated at 37°C under microaerobic conditions 3 days. The mutants were checked for curved shape and motility before tested in assays.

4.3.6 INT407 adhesion assay

INT407 cells (representing intestinal cell line) were grown in MEM (+glutamax) media (Invitrogen) added 25 µg/ml gentamycin and 10% heat inactivated fetal bovine serum in 5 % CO₂. Cells were seeded at 2.5×10^5 pr well in 24 well plates, incubated overnight and checked for 100 % confluent monolayer. The *E. coli* clones were grown overnight in MagicMedia broth at 37°C and *C. jejuni* on blood agar plates microaerophilic at 37°C. Immediately before assay, the OD₆₀₀ of the bacteria was adjusted to 1 in PBS and 1 ml bacteria culture was added the INT407 cells and cells were incubated with bacteria for 2 hours at 37°C, then resuspended and diluted in PBS and spotted on agar plates with appropriate antibiotics.

4.3.7 Electron microscopy

To investigate, whether the *C. jejuni* mutant strain differed morphologically from the wild type strain, a transmission electron microscopy analysis was conducted. Initially, the bacterial cultures were fixated in 1% glutaraldehyde (EMS, Hatfield, USA) for 30 minutes. To improve the adhesion of the bacteria, formvar coated 400-mesh copper grids were treated for 5 minutes with alcian blue (Sigma-Aldrich). The alcian blue treated grids were placed on top of cultures of *C. jejuni* NCTC11168 and *C. jejuni* NCTC11168Δ0034, respectively, and after 5 minutes of incubation, most of the suspensions were removed from the grids with filter paper and the grids were stained for 30 seconds with phosphotungstic acid (BDH Chemicals). The grids were allowed to air-dry, and then they were viewed in a Morgagni 268D transmission electron microscope, and pictures were taken using a Mega-view III digital camera.

4.3.8 Motility assay

Motility assay was carried out to ensure no altered motility for the 11168Δ0034 mutant. 0.25 % soft agar plates were added 1 µl bacterial culture (OD₆₀₀ adjusted to 0.1) in the middle of the plate and diameter was measured over a time period.

4.3.9 Serum resistance assay

Serum sensitivity assays were performed by modification of the method of Blaser et al.,1985. *C. jejuni* strains were grown overnight in Brucella biphasic cultures at 37°C, washed in PBS, pH 7.4, and adjusted to a concentration of 10³ CFU/ml. *C. jejuni* cells (10-µl aliquots) were incubated in 240-µl pools of whole human blood (venous blood), human serum (whole blood incubated at 25°C 30 min, centrifuged 1000xg 10 min at 4°C and supernatant isolated) and heat inactivated human serum (56°C for 30 min) respectively for 30, 60, 90 and 120 min. Following the incubation period, CFU was enumerated on BHI agar.

4.3.10 Biofilm and autoagglutination

Cell-to-cell autoagglutination was assayed in PBS as described by Misawa and Blaser, 2000. Biofilm assay was made in 50 ml centrifuge tubes containing 25 ml inoculated Brucella broth with NTCT 11168 and the 11168Δ0034 respectively. A glass slide was

added each tube and incubated micro aerobic for 48 h. Then the slides were stained with crystal violet and biofilm formation visualised.

4.3.11 Protein purification

His-tag purification was made with the already GST-His-tagged constructed vector from Geneservice. An overnight pre-culture of *E. coli* BL21(DE3) containing the vectors was 50-fold diluted to inoculate 1000 ml LB medium containing appropriate antibiotics. The cultures were incubated with shaking at 37 °C to an OD₆₀₀ of 0.5, then induced with 10 mM IPTG and incubated with shaking for 16 hours at 30 °C. After induction, cells were lysed on ice in 20 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10 % glycerol) by addition of 1 mg/ml lysozyme followed by sonication. Lysates were cleared by centrifugation at 15,000xg for 30 min. Proteins were purified by nickel affinity chromatography using the Ni-NTA resin (Qiagen) equilibrated with lysis buffer and eluted with 250 mM imidazole. Eluted proteins were concentrated and dialyzed against 25 mM HEPES pH 7.5, 50 mM NaCl, 10 % glycerol.

4.3.12 Mouse immunization

To study the proteins ability of protection against infection, immunization of mice were carried out. Due to patent issues, the original nomenclature is not used for the genes in this study. Five proteins were tested for their ability to protect against *C. jejuni* infection; SSI-1, SSI-2, SSI-3, SSI-4, SSI-5 and Cj1228 (HtrA) was also included because of its known immunity and therefore a potential vaccine candidate. Mice (10 in each group) were immunized with 5 microgram/dose, except one with 1.6 microgram/dose (SSI-4), along with adjuvant (GNE, Intervet, NL). Four weeks after, the mice (Balb/c for colonization and CH3/HeN for invasion) were treated for three days with streptomycin (5 g/l in drinking water) and challenged one day later with *C. jejuni* 81-116 (6x10⁵ CFU, colonization study) and 72Dz/92 (5x10⁷ CFU, invasion study) respectively. Balb/c mice 6-8 weeks (female) were used in groups of three. One fresh faecal dropping was collected and weighted from each animal and dilutions were made in order to determine CFU/gram faeces. Faecal samples were collected from the Balb/c mice regularly in 23 days. Necropsy was prepared one week after challenge of the CH3 mice, spleen and liver were collected and CFU/organ measured.

4.3.13 Predictions of protein localization

Prediction of protein localization and amount of transmembrane helices was made by TMHMM 2.0 server (Moller et al., 2001). The SignalP 3.0 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models (Emanuelsson et al., 2007). The LipoP 1.0 server produces predictions of lipoproteins and discriminates between lipoprotein signal peptides, other signal peptides and N-terminal membrane helices in Gram-negative bacteria (Junker et al., 2003). All three servers are available at <http://www.cbs.dtu.dk/services/>.

4.4 Results

4.4.1 Identification of *C. jejuni* antigens.

With the aim of identifying immuno-reactive *C. jejuni* proteins plasmid DNA was isolated from a pooled mixture of commercial library clones established by Parrish et al (2004) in the plasmid pTLJ03 (Parrish et al., 2004). Plasmid DNA was transformed into *E. coli* BL21 to allow expression from the T7 promoter. The resulting transformants were individually spotted on a nitrocellulose membrane and reacted with serum isolated from a rabbit infected with a *C. jejuni* serotype 2. The immuno-screening revealed several immunogenic *E. coli* clones as shown in figure 4.1.

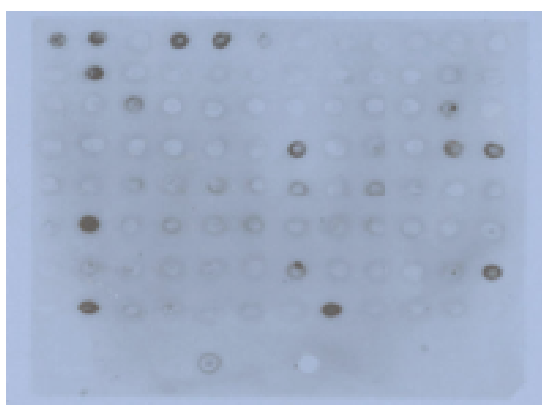


Figure 4.1: Immunoblot assay screen. The figure show a representative immunoblot assay with 96 *E. coli* clones each expressing a *C. jejuni* gene reacting against rabbit serum. Dots with a strong reaction where isolated and the genes sequenced. A total of 52 clones were sequenced representing 25 genes. Lower left dot is the BL21 expression strain with no insert and the right a media control.

Inserts in vectors isolated from the most immunogenic clones were selected for sequencing and from a total of 2304 clones, 52 inserts were sequenced representing 25 genes (table 4.1).

Table 4.1. Sequenced highly immunogenic clones.

Cj number	Gene	Protein ID	Class*	Predicted mass (kDa)
Cj0014c		Putative integral membrane protein	3.C.1	20.3
Cj0034c ⁴		Putative periplasmic protein	3.C.5	26.4
Cj0111 ³		Putative periplasma protein	3.C.5	29.2
Cj0203		Putative transmembrane transport protein	4.A.6	48.2
Cj0383c	<i>ribH</i>	6,7-dimethyl-8-ribityllumazine synthase	1.G.9	16.6
Cj0404 ³		Putative transmembrane protein	3.C.1	30.7
Cj0408	<i>frdC</i>	Fumerate reductase cytochrome B subunit	1.B.3	30.0
Cj0477	<i>rplL</i>	50S ribosomal protein	3.A.2	13.0
Cj0525c ³	<i>pbpB</i>	Putative Penicillin binding protein	3.C.4	68.3
Cj0645 ²		Putative secreted transglycosylase	3.C.4	42.8
Cj0774c ⁵		ABC transport system ATP binding protein	4.A.6	37.9
Cj0811	<i>lpxK</i>	Tetrasyldisaccharide 4'-kinase	3.C.2	36.2
Cj0917c	<i>cstA</i>	Carbon starvation protein A homolog	3.C.1	75.7
Cj0965c		Putative acyl-CoA thioester hydrolase	6.A	14.4
Cj1092c	<i>secF</i>	Protein-export membrane protein	4.E	36.1
Cj1094c	<i>yajC</i>	Preprotein translocase subunit	4.E	10.1
Cj1163c ⁴		Putative cation transport protein	4.A.2	35.8
Cj1174 ³		Putative efflux protein	5.D	11.2
Cj1292	<i>Dcd</i>	Deoxycytidine triphosphate deaminase	1.F.3	20.6
Cj1364c	<i>fumC</i>	Fumerate hydratase	1.B.3	50.7
Cj1371 ²		Putative periplasmic protein (vacJ homolog)	4.1	26.5
Cj1382c ⁴	<i>fldA</i>	Flavodoxin	1.B.7	17.1
Cj1529c ⁵	<i>purM</i>	Phosphoribosylaminoimidazole synthase	1.F.1	35.6
Cj1628	<i>exbB2</i>	Putative exbB/tolQ family Transport protein	4.A.6	15.7
Cj1632c		Putative periplasmic protein	3.C.5	6.0

* Class due to Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/C_jejuni/). Numbers in superscript are the amount they were identified as strong reactors against the rabbit antiserum and sequenced.

Classes:

- 1.B.3 Tricarboxylic acid cycle
- 1.B.7 Electron transport
- 1.F.1 Purine ribonucleotide biosynthesis
- 1.F.3 2'-deoxyribonucleotide biosynthesis
- 1.G.9 Riboflavin
- 3.A.2 Ribosomal protein synthesis and modification
- 3.C.1 Membrane, lipoprotein and porins
- 3.C.2 Surface polysaccharides, lipopolysaccharides and antigens
- 3.C.4 Murein sacculus and peptidoglycan
- 3.C.5 Miscellaneous periplasmic prot.
- 4.A.6 Other cell process
- 4.E Protein and peptide secretions
- 4.I Pathogenicity

Ten of the 25 clones (Cj0034, Cj0203, Cj0404, Cj0525c, Cj0645, Cj0917c, Cj1094c, Cj1371, Cj1382c, Cj1632c) were further tested for reaction against human antiserum Penner serotype 2 and were found positive (figure 4.2). The ten clones were selected either because of a repeatedly strong reaction against the rabbit antiserum (Cj0034c, Cj0404, Cj0525c, Cj0645, Cj1382c), its predicted membrane associated localization (Cj1094c), already existing knowledge from other pathogens (Cj0917c, Cj1371), predicted to be only in *Campylobacter* (Cj1632c) or because of an also altered autoagglutination phenotype (Cj0203).

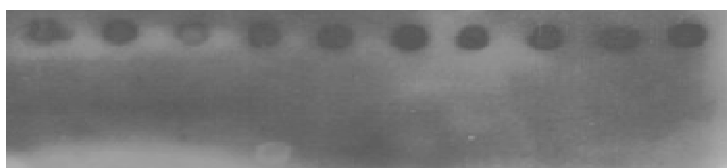


Figure 4.2. Immunoblot assay with human serum. The clones Cj0034, Cj0203, Cj0404, Cj0525c, Cj0645, Cj0917c, Cj1094c, Cj1371, Cj1382c, Cj1632c, from left to right, were tested for its reactivity against the antiserum isolated from a human patient infected with a *C. jejuni* serotype 2. All the clones reacted more or less with the serum. Lower left is a medium control and lower right a BL21(DE3) control (not easy to see).

The genes sequenced from the immunogenic *E. coli* clones, were analysed with the definitions of functional classification given at the Wellcome trust Sanger Institute webpage (http://www.sanger.ac.uk/Projects/C_jejuni/). The 25 genes were distributed over five of the six classification groups. Nine of the 25 genes in group 3 “Macromolecule metabolism” all in subgroup 3.C “Cell envelope”, which further contain the groups; 3.C.1 Membranes, lipoproteins and porins, 3.C.2 Surface polysaccharides, lipopolysaccharides and antigens, 3.C.3 Surface structures, 3.C.4 Murein sacculus and peptidoglycan and 3.C.5. Miscellaneous periplasmic proteins. The Nine genes were mainly found in the subgroups 3.C.1 and 3.C.5. The remaining 16 genes were located in the groups of small molecules metabolism, cell processes (including transport/binding proteins and pathogenicity), and other (drug sensitivity).

4.4.2 Prediction of protein localization

Prediction of localization of the proteins and amount of transmembrane helixes was made by TMHMM 2.0 server (Moller et al., 2001), prediction of signal peptide molecule with SignalP 3.0 server based on hidden Markov models (Emanuelsson et al., 2007) and prediction of lipoproteins with Lipop (Junker et al., 2003).

Results are shown in table 4.2.

Table 4.2. Localization of the proteins and possible signal peptide molecule predicted by TMHMM, SignalP and Lipop

Cj number	Transmembrane Localization	Helixes	Predicted signal peptide (probability)	Lipoprotein
Cj0014c	Inside and outside	3	yes (0.951)	TMH
Cj0034c	Inside and outside	1	yes (0.999)	SpI
Cj0111	Inside and outside	1	no	CYT
Cj0203	Inside and outside	12	no	TMH
Cj0383c	Outside	0	no	CYT
Cj0404	Inside and outside	1	no	CYT
Cj0408	Inside and outside	5	no	TMH
Cj0477	Outside	0	no	CYT
Cj0525c	Inside and outside	1	no	TMH
Cj0645	Outside	0	yes (0.992)	SpI
Cj0774c	Outside	0	no	CYT
Cj0811	Inside and outside	1	no	TMH
Cj0917c	Inside and outside	16	yes (0.940)	TMH
Cj0965c	Inside	0	no	CYT
Cj1092c	Inside and outside	4	no	TMH
Cj1094c	Inside and outside	1	no	CYT
Cj1163c	Inside and outside	6	no	CYT
Cj1174	Inside and outside	4	no	TMH
Cj1292	Outside	0	no	CYT
Cj1364c	Outside	0	no	CYT
Cj1371	Outside	0	yes (1.000)	SpI
Cj1382c	Outside	0	no	CYT
Cj1529c	Outside	0	no	CYT
Cj1628	Outside and inside	3	no	TMH
Cj1632c	Outside	0	yes (0.999)	SpI

TMH: Transmembrane helixes, SpI: Signal peptidase I, SpII: Lipoprotein signal peptidase II, CYT: cytoplasmic or all the rest.

14 out of 25 proteins are predicted to contain one or more membrane helixes, two of them further with a signal peptide. The location of ten proteins is predicted to be outside, where three of them harbor a signal peptide. None of the proteins were predicted to contain a lipoprotein signal peptidase.

4.4.3 Virulence detection by use of cell adhesion assay

For 10 selected *E. coli* clones INT407 cell line adhesion assay was performed in triplicates (figure 3.3). The assay showed an enhanced adhesion ability of several of the clones (Cj0034c, Cj0404, Cj1371).

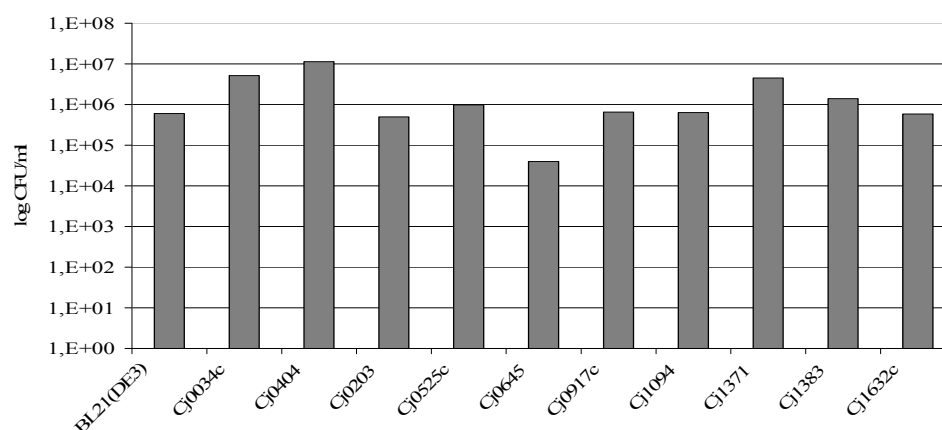


Figure 4.3. INT407 cell line assay. CFU/ml for *E. coli* clones expressing the respective genes.

Subsequently, gene-specific *C. jejuni* mutants, were tested in the same cell adhesion assay. For that purpose mutations were first successfully transferred from NCTC 11168H to NCTC 11168 by natural transformation and checked for curved shape and motility. The cell adhesion assay showed a reduced ability to adhere to INT407 cells for the mutant, Cj0034c (figure 4.4).

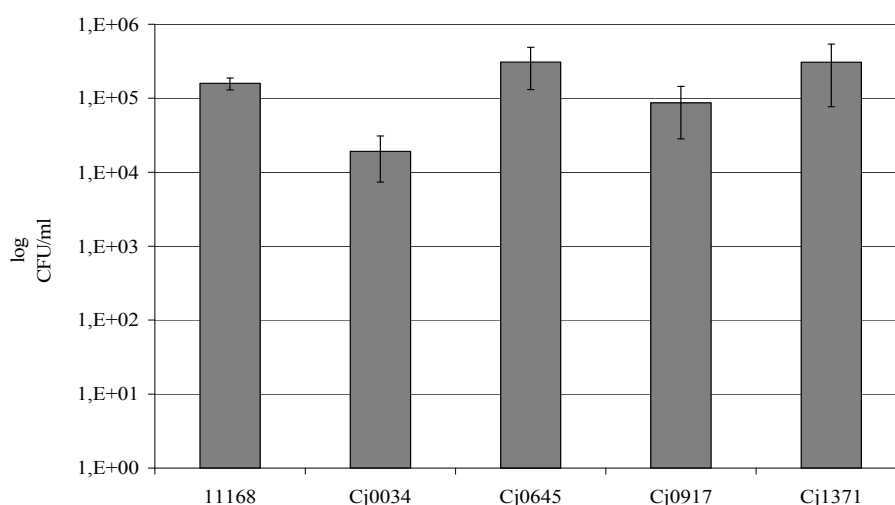


Figure 4.4. INT407 cell line assay. CFU/ml of knock out mutants of *C. jejuni* 11168 Δ Cj0917, Δ Cj0034c, Δ Cj1371, and Δ Cj0645 compared to the NCTC 11168 wildtype. Standard bars indicate standard deviations.

We further confirmed, by means of electron microscopy, that the mutation in Cj0034 did not result in major structural changes of the bacterial cell morphology (figure 4.5).

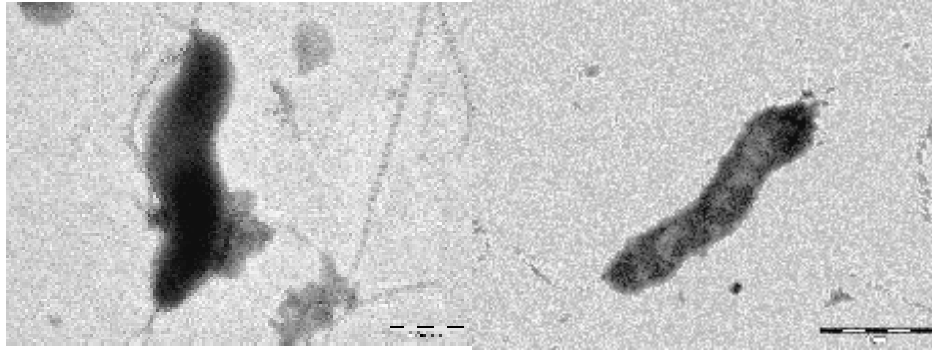


Figure 4.5. Electron microscopy of *C. jejuni* NCTC 11168 left and *C. jejuni* NCTC11168Δ0034 right

In addition, serum resistance, motility, auto agglutination and biofilm formation, were determined but no differences were observed compared to wild type for the *C. jejuni* mutant.

4.4.4 Mouse immunization

Several proteins were selected to test as vaccine components in two *Campylobacter* oral challenge mouse models; one in C3H/HeN mice in which invasion in liver and spleen was measured and the other in Balb/c mice in which shedding faeces was determined. The challenge study (figure 4.6) showed a reduced invasion into spleen and liver for at least two of the proteins; SSI-2 and SSI-3. Challenge colonization results are shown in figure 4.7, where no decreased colonization for any of the proteins was observed.

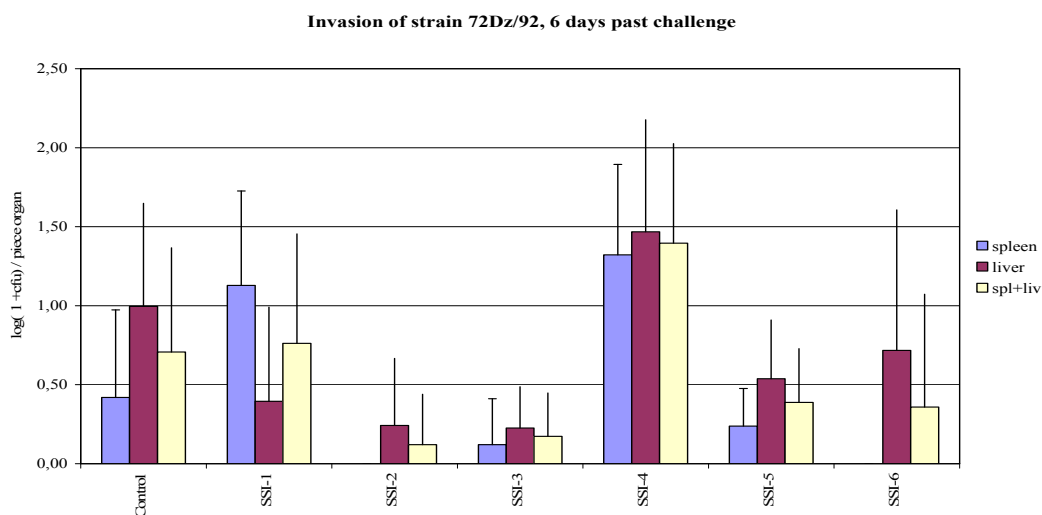


Figure 4.6. Challenge invasion study. Invasion of *C. jejuni* 72Dz/92 in spleen and liver of C3H/HeN mice, 6 days after challenge. Values determined individually, log-transformed (log 1+CFU) and calculated as averages per group. Error bars indicate standard deviation. The mice were immunized with SSI-1 to SSI-6 before challenge.

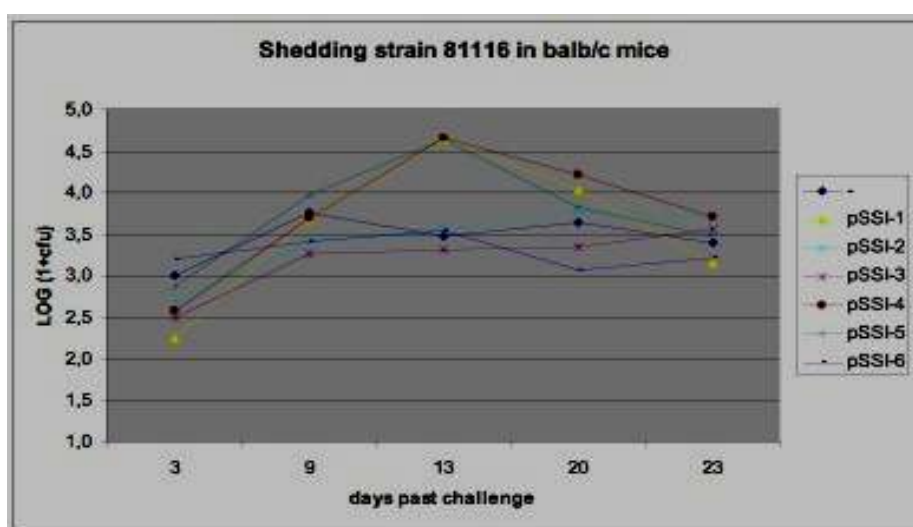


Figure 4.7. Challenge colonization study. Colonization of Balb/c with *C. jejuni* strain 81116 after challenge. Individual colonization was determined per 0.2 fecal pellet, lot-transformed and calculated as average per group. The mice were immunized with SSI-1 to SSI-6 before challenge.

4.5 Discussion

In this study we have successfully identified immuno-reactive proteins of the important human pathogen, *C. jejuni*. An *E. coli* library expressing single *C. jejuni* proteins was screened using specific antibodies from rabbit resulting in the selection of immuno-reactive proteins among the total amount of antigens in *C. jejuni*.

Identification of surface structures is important to understand the virulence of *C. jejuni* particular in relation to interaction with the host. Mechanisms like colonization, adhesion and invasion, biofilm formation and immune evasion are essential for the pathogen to establish a successful infection. We identified proteins predicted to be surface related by the classification of Sanger Institute and bioinformatical predictions. 14 proteins were predicted to contain one or more membrane helices, three of them with predicted signal peptide. Another three proteins were predicted to maintain a signal peptide and only one protein was predicted to solely be located inside the cell. Based on predictions, the majority of the proteins are exposed to the surface and these findings back up the validity of our approach aimed at isolation of novel surface proteins.

Several other studies have been published with the aim to identify these structures. The work by Cordwell et al (2008) identified proteins using 2-DE/MS and 2-DLC/MS/MS

found several proteins also identified in this study (Cj0034c, Cj0404, Cj0917c, Cj0774c, Cj1092, and Cj1094). A study by Prokhorova et al (2006) used proteomics for the detection of various *Campylobacter* surface proteins and demonstrated that some of them further were protective in a *C. jejuni* colonization model. They did, as in this study, identify flavodoxin (Cj1382c) with high significance of identification but the protein was not studied in the mouse vaccination-challenge experiment. Flavodoxin was also identified in a proteomic analysis by Seal et al (2007), where proteins expressed in a robust chicken colonizer strain were compared with a poor chicken coloniser strain. Flavodoxin was along with Cj1292 (deoxycytidine triphosphate deaminase) identified in the high colonizer strain. A study by Garenaux, et al (2008) found the flavodoxin protein to be over-expressed after exposure to paraquat, suggesting the protein could play a role in *C. jejuni* oxidative stress resistance. Kaakoush et al (2007) identified eighteen genes bioinformatically as potential contributors to the differences in oxygen tolerance between strains. Cj0203 was, among others, considered the top potential contributor and the gene was over expressed along with important surface adhesins such as CadF and FlaA, indication a potential role of oxygen as trigger of transcription of these genes.

In this study, we found several interesting and potential important genes and because of altered adhesion ability, we choose further investigations of the Cj0034c. It was found to be important for adhesion to INT407 epithelial cells by a reduction of adhesion of the Cj0034c mutant, which also correlated with the *E. coli* INT407 cell assay where Cj0034c showed some enhanced adhesion to the cells. Therefore, it could have a correlation to and importance for virulence. The gene is grouped in the 3.C.5 Miscellaneous periplasmic proteins together with other very important antigenic and virulence genes, such as PEB2, 3 and 4. Cj0034c is a 26.4 kDa putative periplasmic protein found in other *C. jejuni* subspecies. It was predicted to contain one helix and one signal peptide, which most likely makes it surface exposed and therefore a target for the immune system. Furthermore does the outside exposure it likely that it contributes to host interaction by adhesion to epithelial cells.

Vaccination challenge experiments showed that SSI-2, SSI-3 and SSI-5 resulted in the

best protection against invasion in spleen and liver. SSI-2 is a large antigen, not likely to be expressed by a vaccine strain. SSI-5 is a smaller antigen that might be suitable for expression in the vaccine strain but SSI-3 is also a small molecule and was easily produced by *E. coli* BL21(DE3) during purification. In addition, SSI-3 gave the best protection against invasion and some against colonization. Therefore we propose that SSI-3 is a good vaccine candidate against *C. jejuni*.

4.6 Acknowledgements

The authors thank Brendan Wren for providing the *C. jejuni* mutants, David Ussery and Carsten Friis for support with prediction of protein localization, Ruud Verstegen for technical assistance during immunization experiments, and Marian Jørgensen for critical manuscript proof reading. This project is funded by Statens Serum Institut, University of Copenhagen and FOBI Research School.

Chapter 5. Screening for virulence associated phenotypes of *Campylobacter jejuni*.

5.1 Abstract

Campylobacter jejuni has been identified as the leading cause of food-borne bacterial gastroenteritis in the developed world, including Denmark. Compared to other important pathogens, less is understood concerning the virulence factors of this human pathogen. It is well established that the surface of *C. jejuni* is crucial for its ability to colonize, persists and cause disease in humans. Surface structures have been identified in *C. jejuni* to be important for adhesion to epithelial cells and/or biofilm formation and further found also to play a role in virulence development. The aim of this work was to identify novel proteins involved in cell-cell contact (autoagglutination), biofilm formation and adhesion to epithelial cells using an *E. coli* open reading frame library expressing genes from *C. jejuni* NCTC 11168.

5.2 Introduction

Campylobacter jejuni is the leading cause of food-borne bacteria diarrhoea in the developed world, including Denmark with 3352 cases in 2009. The major source of infection is consumption and handling of contaminated and undercooked poultry products. Infection with this organism can lead to acute illness as well as serious life-threatening consequences, such as Guillain-Barré syndrome (Allos, 1997). Even though diarrhoeal infection with *C. jejuni* is more frequent than *Salmonella*, understanding of *C. jejuni* pathogenesis and the critical virulence factors is far from understood.

Autoagglutination (AAG) has been known as an indicator for virulence in other gram-negative pathogens, including *Vibrio cholera*, *Bordetella pertussis*, *Neisseria gonorrhoeae* and *Yersinia* (Chiang et al., 1995; Menozzi et al., 1994; Swanson et al., 1971; Laird and Cavanaugh, 1980). To date, the role AAG in *C. jejuni* pathogenesis has not been determined but associations with adherence and invasion and a strong association to surface proteins and the flagella have been found (Misawa and Blaser, 2000). In addition, studies have shown a relation between AAG and flagellin glycosylation (Golden and Acheson, 2002; Guerry et al., 2006). AAG has furthermore

been found to be dependent on quorum sensing (Jeon et al., 2003). Biofilm, or cell surface associated growth, is another form of cell community growth, which was found important for environmental stress resistance, such as desiccation and antimicrobials, as well as increased resistance to host defences in a number of bacterial pathogens including *C. jejuni* (Davis et al., 2002). In *C. jejuni*, the flagella has been found to be implicated in biofilm formation but the complete molecular understanding is not established. Adhesion to epithelial cells is crucial for *C. jejuni* and its interaction with the host and some adhesion factors have been studied, including MOMP (Moser et al., 1997), PEP1 (Leon-Kempis et al., 2006), Omp50 (Bolla et al., 2000), lipoproteins Omp18 (Burnens et al., 1995, Konkel et al., 1996), JlpA (Jin et al., 2001) and Cia proteins (Rivera-Amill et al., 2001). *C. jejuni* has in previous studies been found positive for haemolytic activity depending on culture conditions (Arimi et al., 1990; Misawa et al., 1995). So far, two genes in *C. jejuni* have been associated with haemolytic activity: *ceuE* and *pldA*. The CeuE protein is a lipoprotein that functions as a periplasmic binding protein. It is a part of an ABC transport system associated with enterobactin transport across the inner membrane (Stintzi et al., 2008). *pldA*, encoding phospholipase A, has been studied for *C. coli* and found to contribute to cell-associated haemolysis (Grant et al., 1997). A homologue of *pldA* is also present in the genome of *C. jejuni* NCTC 11168 (Parkhill et al., 2000).

In this study, the aim was to identify genes important for adhesion, persistence and virulence in *C. jejuni* by screening a commercial *E. coli* Open Reading Frame (ORF) library.

5.3 Material and methods

5.3.1 Strains, plasmids and growth conditions

The bacterial strains used in this study included *E. coli* SURE (Stratagene) and *E. coli* BL21(DE3) (Stratagene) and the plasmid was pTLJ03 (Parrish et al., 2004). Strains and plasmid originates from a *C. jejuni* ORF library created by Parrish et al (2004) available from Geneservice. The expression clone set comprises of >1,600 *C. jejuni* ORF's and the expression vector pTLJ03 generates N-terminal GST-His-tagged fusion proteins. Strains were grown in LB media or the expression media MagicMedia (Invitrogen) at

37°C. Clones containing pTLJ03 containing strains were grown in media containing 50 µg/ml ampicillin unless otherwise specified. The library was originally created in *E. coli* SURE for optimal storage. The strain does not contain the T7 polymerase and for that reason the library was transformed to the *E. coli* BL21(DE3) expression strain. The clones were grown separately in microtiter plates in 200 µg LB media containing ampicillin overnight and subsequently the plasmids were purified as a pool and transformed to the chemocompetent *E. coli* BL21(DE3) strain. This revealed an expression library consisting of 2304 clones (24 microtiter plates). The variability of the clones was verified by comparison of protein expression of randomly selected clones on a SDS-PAGE gel.

5.3.2 Screening for autoagglutination

Autoagglutination assay for *E. coli* clonal library was studied in microtiter plates. *E. coli* BL21(DE3) clones were grown and induced over night in 200 µl MagicMedia (Invitrogen) in microtiterplates and the next day the wells were inspected by visualisation for autoagglutination. Only the clones with clear and high amount of autoagglutination were considered positive. Negative controls of *E. coli* BL21(DE3) were included.

5.3.3 Biofilm formation in microtiter plates

Screening for biofilm formation was performed in microtiter plates in MagicMedia (Invitrogen) containing appropriate antibiotics. A replicator was used to inoculate 3 sterile 96-well flat/bottom microtiter plates in succession. The inoculated plates were then grown at 37°C for 24 hours, after which the media was discarded. The wells were then washed twice with 200 µl Phosphate-Buffered Saline (PBS) and 150 µl 0.1% crystal violet was added to each well and discarded after 15 min. Then washed with 200 µl PBS twice. The remaining crystal violet was dissolved in 150 µl 96% ethanol for 30 min. An OD₆₂₀ reading was made on ELISA reader and the average for each well was determined. Negative controls of *E. coli* BL21(DE3) were included.

5.3.4 HeLa cell line adhesion assay

Adhesion to epithelial cells was studied using a HeLa cell line kindly provided from the

Department of Virology, Statens Serum Institut as a monolayer. DME media (Invitrogen), supplemented with 10% heat inactivated fetal bovine serum and antibiotics, was disposed and the wells were washed three times with 0.5 ml PBS for 5 min at 100 rpm. 0.5 ml fresh DME media without antibiotics was then added each well. *E. coli* BL21(DE3) ORF clones were grown and thereby induced overnight in MagicMedia (Invitrogen) and the next day diluted to 10^{-2} . 12.5 μ l bacterial culture was added to each well and the plates were left at 37 °C for 3 hours. After inoculation, each well was rinsed three times with PBS. Adherent bacteria were released by the addition of 200 μ l 0.5% Triton X-100 (Sigma). PBS was then added and adherent bacteria were quantified by plating appropriate dilutions on LB agar medium. Control wells were prepared in a similar manner but without treatment as a number of total cells. Specific adhesion to HeLa cells was expressed by the percentage of number of CFU adhering to HeLa cells minus total number of CFU.

5.3.5 Haemolysis

The *E. coli* ORF library was screened for hemolytic activity. Defibrinated sheep blood was washed in PBS, centrifuged at 400×g in 5 min at 4°C. This process was performed 3 times. Erythrocyte suspension were adjusted to 16% (8% of total volume when added to each well) with PBS supplemented with 0.1% BSA. 100 μ l MagicMedia was added each well in microtiter plates. *E. coli* clones from the ORF library were transferred from master plate wells to three replica wells using replicator. Plates were incubated for 24 hours at 37 °C under a gas mixture of 92% N₂, 5% CO₂ and 3% O₂ under humid conditions. 100 μ l of 16% washed sheep blood (0.1 % BSA) was then added to each well. After incubation, the plate was centrifuged at 2.200×g for 20 min, supernatant (100 μ l) was taken from each well and transferred to another microtiter plate for OD₅₅₀ measurement in a microplate reader. Wells with the highest values were rerun with 6 replicas using this procedure.

5.3.6 Growth in chicken mucus

Mucus was recovered from the intestines of eleven 30 days old chickens kindly provided by the hatchery DanHatch. The chickens were fasted for 1 day before the intestines were obtained. Three sections of the intestines were isolated; jejunum, ileum

and the two colic ceca (figure 5.1). The sections were placed in petri dishes containing PBS and kept on ice. The intestines were cut open and washed with buffer, and each section was transferred to a petri dish with fresh PBS buffer, where the mucus was scraped off the intestine walls.



Figure 5.1. Isolation of chicken intestine mucus. Three sections of the chicken intestine were isolated; jejunum, ileum and ceca.

All the scraped material from each type of section was centrifuged 15 minutes at 10,000x g, and the supernatants were recovered. The mucus was checked for possible *Campylobacter* contamination by plating on blood plates and CCDA plates and incubated at 37°C and 42°C, for three days. The mucus was stored at -20°C. Prior to use, the mucus was sterilized by treatment with UV-light for 15 minutes and tested for contamination on LB agar plates, finally, the protein concentration was measured and adjusted to 1 mg/ml.

Screening of clones with enhanced growth ability in chicken mucus was studied by inducing the clones individually in MagicMedia, and then inoculating mucus from the three isolated intestinal sections with a pool of 96 clones. The pool of clones, inoculated in the three sections respectively, was grown overnight and present clones isolated in a dilution row. Three clones, from each experiment, were isolated from the highest dilution of an overnight culture, the plasmid purified, as described in section 4.3.6 and then ran on a 1% agarose gel. The screening was made for 288 clones (three times 96 clones).

5.3.7 Plasmid purification and sequencing

Plasmids from overnight cultures grown in LB media were purified using a QIAfilter™ Plasmid Midi Kit and plasmids were sequenced by Eurofins mwg operon, using their Value Read service.

5.3.8 Prediction of protein localization

Prediction of protein localization and amount of transmembrane helices was examined by TMHMM 2.0 server (Moller et al., 2001). The SignalP 3.0 server predicts the presence and location of signal peptide cleavage sites in amino acid sequences. The method incorporates a prediction of cleavage sites and a signal peptide/non-signal peptide prediction based on a combination of several artificial neural networks and hidden Markov models (Emanuelsson et al., 2007). The LipoP 1.0 server produces predictions of lipoproteins and discriminates between lipoprotein signal peptides, other signal peptides and n-terminal membrane helices in Gram-negative bacteria (Junker et al., 2003). All three servers are available at <http://www.cbs.dtu.dk/services/>.

5.4 Results

To identify genes important for *C. jejuni* virulence and/or interaction with the host, several assays with an *E. coli* ORF library were used, including autoagglutination assay, biofilm formation assay, HeLa cell adhesion assay and haemolysis assay.

5.4.1 Screening for autoagglutination positive clones

A commercial ORF library was screened for autoagglutination in microtiterplates (figure 5.2). A total of 2304 clones were screened and 41 wells were found positive for autoagglutination. The result was solely achieved by visualization.

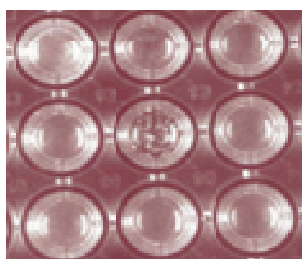


Figure 5.2. Screening for autoagglutination. The screening for autoagglutination was made in microtiter plates with overnight induced *E. coli* BL21(DE3) containing plasmid with various gene inserts. The well in the middle represent a strong autoagglutinating clone.

Of the 41 identified clones, 7 genes were successfully sequenced and identified. The identified genes were: Cj0108, Cj0121, Cj0125, Cj0203, Cj0434, Cj1223 and Cj1717.

5.4.2 Screening for biofilm formation

In order to identify *E. coli* clones with altered phenotype according to biofilm

formation, a screening of the total library (2304 clones) in microtiter plates was made. The clones were induced overnight and the amount of biofilm was measured in microtiter plates by visualisation and OD measurement. Several clones where reproducible found to form biofilm (figure 5.3), and by sequencing four of these clones were found to encode the genes Cj0525c, Cj0774c, Cj0034 and Cj0404.

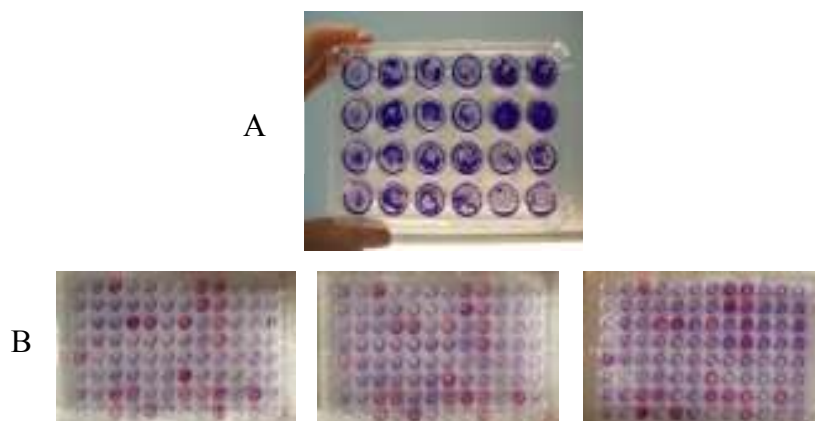


Figure 5.3. Screening for biofilm formation. Screening for biofilm formation was made in microtiterplates. A: Biofilm formation in larger wells with selected clones producing relatively strong biofilm. B: Microtiter plates from the initial screening with 3 replica showing the reproducibility.

5.4.3 HeLa cell adhesion assay

Adhesion to epithelial cell is recognised as being important for the pathogenesis of *C. jejuni* in the human host. A HeLa cell adhesion assay was used in order to identify *C. jejuni* adhesion factors, which could promote cell adhesion in an *E. coli* background. A total of 288 clones were screened for adhesion to HeLa cells. Clones showing improved adhesion were investigated in a secondary experiment where growth rate was taken into account. Figure 5.4 shows the results for the secondary experiment.

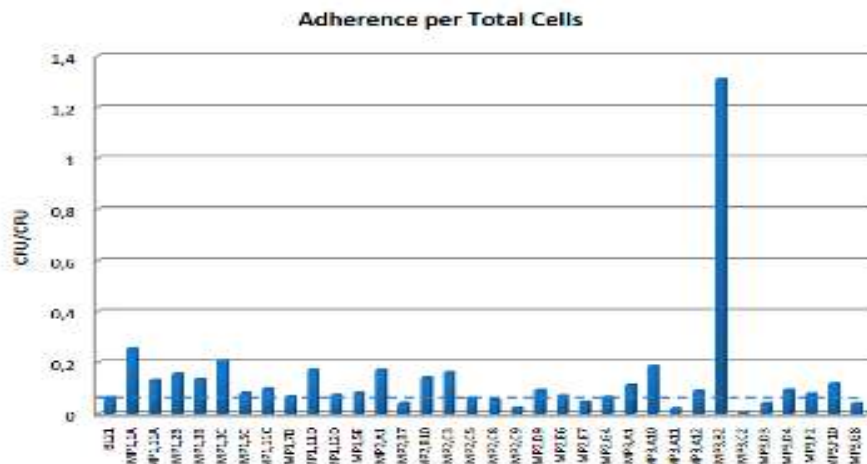


Figure 5.4. HeLa cell adhesion assay. Adhesion to HeLa cells was initially tested for 288 clones. Of them 33 clones with enhanced adhesion were tested in a secondary experiment. Y-axis shows CFU/CFU and X-axis the different clones. 12 clones adhered twofold or more to the HeLa cells compared to the control (BL21(DE3) without insert).

The HeLa cell line assay revealed 12 clones that adhered double or more the value of the control (BL21(DE3) without insert). Because of sequencing problems, only one clone was identified as Cj0408 by sequencing. The implausible value of 1.3 CFU adhering per CFU in the well must be ascribed to deviations in the assay.

5.4.4 Haemolysis assay

Haemolysis was measured with a microplate reader and results are shown at figure 5.5. It illustrates the OD₅₅₀ values of the wells of a) microtiter plate (Mp) 1,2 and 3 b) OD₅₅₀ values from Mp 4 to 12 and c) OD₅₅₀ values from 13 to Mp 21.

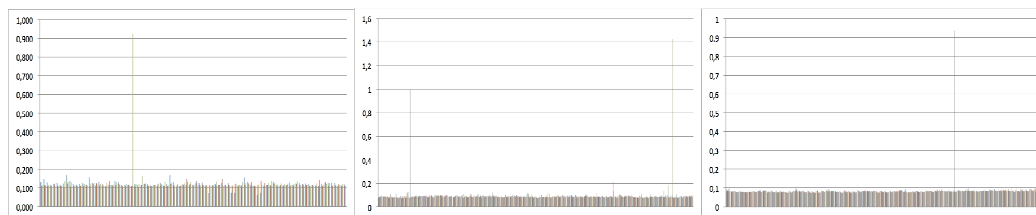


Figure 5.5. Haemolysis assay. Haemolysis was measured on a microplate reader as the amount of lysed defibrinated sheep blood when incubated with different *E. coli* BL21(DE3) expressing genes from *C. jejuni*. Y-axes are showing the OD₅₅₀ values and the wells with the 2304 clones on the X-axis.

Clones with a high reaction were repeated with 6 replicas with following result:

Microtiter plate	well	OD ₅₅₀ value
Mp1	A2	0.120
Mp1	A6	0.115
Mp2	A8	0.118
Mp3	H4	0.117
Mp10	F8	0.109
Mp10	F10	0.103
Mp12	D3	1.433
Mp12	C3	0.137
Control		
<i>E. coli</i> BL21(DE3) with empty pTLJ03		0.102

One clone (Mp12D3) showed a promising result and was subsequently inoculated on a plate with LB agar (100 µg/ml amp) and incubated at 37°C for 24 hours. 4 colonies were isolated and all tested negative for haemolytic activity. Therefore a contamination was suspected and microscopy confirmed the fact. Because of the contamination and the in general low values and small differences between the *E. coli* BL21(DE3) with empty pTLJ03 and the *E. coli* clones in the experiment, we chose to stop further experiments.

5.4.5 Selection of clones after growth in chicken mucus

96 *E. coli* BL21(DE3) clones were pooled and grown in three sections of mucus isolated from cecum, jejunum, and ileum respectively. This was made to examine if some of the clones would be selected due to enhanced growth abilities in chicken mucus. Three clones were isolated from the highest dilution of an overnight culture. Figure 5.6 show the results obtained in the three sections of mucus.

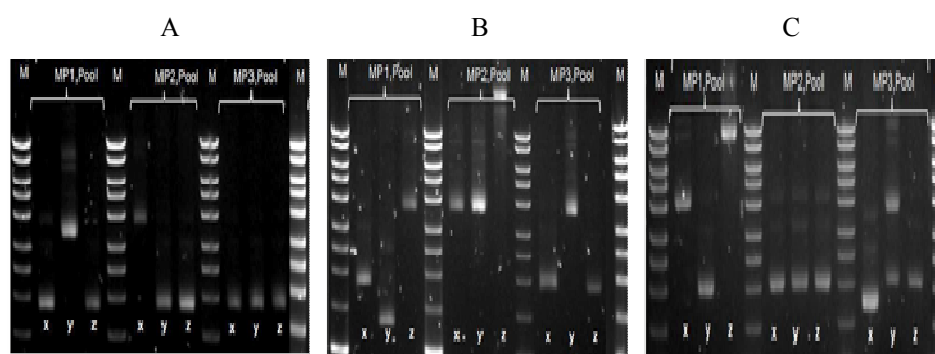


Figure 5.6. Selection of *E. coli* BL21(DE3) clones in chicken mucus. Pool of 96 *E. coli* BL21(DE3) clones, were grown overnight in three different sections of chicken intestinal mucus. A: Mucus isolated from cecum with purified vectors from three clones (x, y and z) in three pools (MP1, 2, 3). B: Mucus isolated from ileum with purified vectors from three clones (x, y and z) in three pools (MP1, 2, 3). C: Mucus isolated from jejunum with purified vectors from three clones (x, y and z) in three pools (MP1, 2, 3).

No obvious selection of the clones was observed, except in the mucus from jejunum with clones from pool two (MP2). It was not possible to sequence the gene for this clone

5.4.6 Localization of proteins and amount of membrane helixes

Prediction of localization of the proteins and amount of transmembrane helixes was made by TMHMM 2.0 server (Moller et al., 2001), prediction of signal peptide molecule with SignalP 3.0 server based on hidden Markov models (Emanuelsson et al., 2007) and prediction of lipoproteins with LipoP (Junker et al., 2003). Clones with altered phenotype in AAG assay and the predictions of protein localization and amount of helixes are shown in table 5.1.

Table 5.1. Localization of the proteins and possible signal peptide molecule predicted by TMHMM, SignalP and LipoP

Cj number	Localization	Transmembrane helixes	Predicted signal peptide	Lipoprotein
Cj0108	Outside	0	no	CYT
Cj0121	Outside	0	no	CYT
Cj0125c	Inside	0	no	CYT
Cj0203	Inside and outside	12	no	TMH
Cj0434	Outside	0	no	CYT
Cj1223c	Outside	0	no	CYT
Cj1717c	Outside	0	no	CYT

TMH: Transmembrane helixes, SpI: Signal peptidase I, SpII: Lipoprotein signal peptidase II, CYT: cytoplasmic or all the rest.

The clones isolated in biofilm assay and HeLa cell adhesion assay where Cj0034c, Cj0404, Cj0408 Cj0525c and Cj0774c, examined in chapter 4, where it were predicted that Cj0034c, Cj0404 and Cj0525c contained one or more helixes. Cj0774c was predicted not to contain a helix but to be localized outside the cell but without signal peptides.

5.5 Discussion

C. jejuni is a common cause of bacterial food-borne disease, but the pathogenetic mechanisms are far from understood. However, it is shown in studies that AAG is associated with virulence. By use of a commercial ORF libray, expressing genes from *C. jejuni* NCTC 11168, we identified several genes that could be important for AAG, biofilm formation and adhesion in *C. jejuni*.

FlaA has in previously studies been recognised as required but not sufficient for autoagglutination and other therefore factors may be involved (Golden and Acheson, 2002). We screened a commercial ORF library and identified 7 genes with possible associations to AAG. The genes were Cj0108 (*atpC*, ATP synthase subunit epsilon), Cj0121 (putative metalloprotease), Cj0125c (DnaK suppressor protein), Cj0203 (putative citrate transporter), Cj0434 (pgm, phosphoglycerate mutase), Cj1223c (*dccR*, two component regulator) and Cj1717c (isopropyl malate isomerase *leuC* large subunit). Cj0203 has also been identified in a previously study screening for antigens of *C. jejuni* (chapter 4). The proteins were predicted to be localised outside except Cj0125c, that was predicted to be inside. One (Cj0203) was predicted to contain a helix. Cj1223c is a part of the two-component signal transduction system with Cj1222c, designated *dccR-dccS*. MacKichan et al (2004) showed that a mutant in both genes, respectively, reduced colonization and inflammation of mice with limited flora but with no altered phenotypes *in vitro* assays. This indicates that the DccR (Cj1223c) response regulator may be required for growth within an *in vivo* environment. The genes identified in AAG study are mainly associated with energy production, metabolism and transport and a direct coupling with AAG is difficult. Other studies have likewise identified genes, beside *flaA*, to be associated with AAG. Golden and Acheson (2002) identified, by use of random transposon mutagenesis, Cj1318, Cj1333, Cj1340 and Cj1062 (putative CinA like protein) as being associated with AAG besides FlaA. Cj1318, Cj1333 and Cj1340 are clustered together and analysis of the surrounding DNA revealed genes involved in sialylation (Golden and Acheson, 2002). In order to verify the obtained results, the AAG positive clones should be repeated in a larger scale, such as glass tubes with a larger amount of volume and the possibility to measure OD of the supernatant.

The screening for biofilm formation lead to several clones able to form biofilm but, due to sequencing problems, only few were identified; Cj0034 (putative periplasmic protein), Cj0404 (putative transmembrane protein), Cj0525c (*pbpB*, putative penicillin binding protein), Cj0774c (ATP transport system, ATP binding protein). The genes were also identified in the screening for antigens (chapter 4) and results were imported

from the previously study described in chapter 4. That is probably the reason for the high number of antigens compared to the total number of clones producing biofilm. On the other hand, it is not unreasonable to suggest that proteins reacting with the immune system could be exposed to the surface of the bacteria and furthermore being expressed during infection. Therefore they could also be important for biofilm formation during colonization and persistent in the gastrointestinal track of the human host.

Adhesion to epithelial cell is essential for *C. jejuni* and far from all adhesion factors, is known. We screened 288 *E. coli* clones for an enhanced ability to adhere, compared to the control and found one clone with a significantly improved adherence; Cj0408 (*frdC*), a fumerate reductase. The gene is a part of the enzyme fumerate reductase which is a membrane bound enzyme catalyzing the reduction of fumerate (Weingarten et al., 2009). There is no apparent link between adhesion to epithelial cell and the function of the enzyme, other than it is membrane bound and probably exposed to the surface and therefore could have a secondary function as adhesion factor.

In general, it is hard to draw a direct line from these studies only made in *E. coli* to virulence associations in *C. jejuni*. But it can be indicated that high expressions of a particular protein could alter the behaviour of *E. coli* with gene insert compared to the control *E. coli* with no insert. An observed altered behaviour should of cause be studied in the originally bacteria, *C. jejuni*, before any conclusions on importance for virulence or host associations can be made. This leads to the perspectives and future work in this study, where specific mutants in NCTC11168 will be made and further tested in the same assays. Furthermore, additional work should be made in order to optimise the sequencing PCR and thereby be able to identify other genes with possible importance for virulence.

We did identify genes that caused altered phenotypes in *E. coli* BL21(DE3) expressing various genes from *C. jejuni* compared to the *E. coli* BL21(DE3) without insert. The genes were found in autoagglutination assay, biofilm formation assay and adhesion assay. Our results suggest that specific virulence genes can be identified by using this clonal library. Due to sequencing difficulties and a limit amount of time, these genes

and their function in *C. jejuni* are not studied in further details.

5.6 Acknowledgements

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Chapter 6. Identification of a fibronectin binding protein of *Campylobacter jejuni*

6.1 Abstract

Campylobacter jejuni is one of the most frequent bacterial causes of food borne gastrointestinal disease in developed countries. Previous studies indicated that binding of the bacteria to human intestinal cells is crucial in host colonization and disease. Fibronectin (Fn) is a 250 kDa glycoprotein abundant in the regions of cell-to cell contact in the gastrointestinal epithelium, where it mediates cell-cell contact and serves as a potential binding site for pathogens. It has earlier been shown that *C. jejuni* harbours two Fn binding proteins, CadF and FlpA and one putative Fn binding protein, Cj1349c. We have investigated the Cj1349c, which was both expressed and studied in *E. coli* and expressed and purified from *E. coli*, for its ability to bind Fn using an ELISA assay. The assays revealed a binding of Cj1349c to Fn and based on the data; we conclude that the Cj1349c protein has a role in attachment to host epithelial cells via Fn binding.

6.2 Introduction

Campylobacter jejuni is the leading cause of food borne human gastroenteritis in developed countries. Infection results in symptoms ranging from mild and watery diarrhoea to more severe diarrhoea with blood and leukocytes. The ability of *C. jejuni* to adhere and colonize the gastrointestinal tract of humans is proposed to be essential for disease (Wassenaar and Blaser, 1999). Cell adherence is multifactorial and a number of adhesins have been identified, including JlpA (Jin et al., 2001), PEB1 (Pei et al., 1998), CapA (Ashgar et al., 2007), CadF (Konkel et al., 1997) and FlpA (Cj1279c) (Konkel 2010). The target of CadF and FlpA was found to be Fn, a component of the extracellular matrix (Konkel et al., 1997, Konkel et al., 2010).

Fn is a glycoprotein formed by two nearly identical 250 kDa subunits. The subunits are linked by disulfide bonds near their C termini. Each Fn monomer is composed of three types of repeating units; type I (~45 amino acids), type II (~60 amino acids) and type III (~90 amino acids) and each monomer contains 12 type I repeats, two type II repeats and

between 15 and 17 type III repeats (Pankov et al., 2002). Fn is involved in many interactions, including tissue repair, embryogenesis, blood clotting, and cell migration/adhesion. Two forms of Fn exist: the soluble plasma Fn synthesised by hepatocytes and the insoluble Fn involved in host cell extracellular matrix (ECM) interaction synthesised by chondrocytes, fibroblasts, endothelial cells, macrophages and some epithelial cells (Pankov et al., 2002). Fn serves as a eukaryotic molecule connecting cells to ECM components, such as collagen and other proteoglycan substrates. The abundance of Fn in regions of cell-to-cell contact in the gastrointestinal epithelium makes it a potential binding site for intestinal pathogens. The invasion of Fn binding bacteria is potentially enhanced by the interaction of Fn and $\alpha_5\beta_1$ integrins, which stimulate rearrangement in the cytoskeleton and membrane protrusion that facilitate the process of internalization and spreading of the bacteria (Gilcrease, 2007).

Several bacterial pathogens are known to bind Fn, including *Staphylococcus aureus* (Kuusula et al., 1978), *Streptococcus pyogenes* (Myhre and Kuusula, 1983), *Salmonella enteritidis* (Baloda et al., 1985), *Escherichia coli* (Fröman et al., 1984), *Neisseria gonorrhoeae* (van Putten et al., 1998), *Mycobacterium avium* (Schorey et al., 1996) and *C. jejuni* (Kuusula et al., 1989; Konkel et al., 1997; Konkel et al., 2010), which emphasises the importance of investigating the mechanism of Fn binding. Besides to the known proteins, CadF and FlpA, an additional putative Fn binding protein (Cj1349c) was annotated in *C. jejuni* Parhill et al., 2000). In this study, we investigate this protein for its ability to interact with Fn.

6.3 Material and methods

6.3.1 Bacteria and growth conditions

E. coli BL21(DE3) (Cj1349c-pET24b(+)) and BL21(DE3) (Cj1349c-pTLJ03) (Parrish et al., 2004) were grown aerobically on or in Luria-Bertani (LB) plates or broth supplemented with ampicillin (Amp) (100 µg/ml). *Campylobacter* strains were cultured on Mueller-Hinton agar plates supplemented with 5 % bovine blood under microaerobic conditions (5 % O₂, 10 % CO₂, 85 % N₂) at 37 °C for 48 hours.

6.3.2 Fibronectin binding assay with glass cover slides

Cover glass slides were coated with 40 µl bovine Fn (10 µg/ml) (Sigma) and air-dried over night. The cover glass slides were washed in PBS and incubated with BSA for 1 hour and washed with PBS again. *E. coli* BL21 (Cj1349c-pTLJ03) and *E. coli* BL21 (Cj1279c-pTLJ03) were induced over night in MagicMedia (Invitrogen) at 37 °C. 0.5 ml overnight cultures were adjusted OD₆₀₀ = 1.5 in PBS and added the cover glass slides coated with Fn. *C. jejuni* were grown microaerobic at 37 °C for 48 hours, then adjusted OD₆₀₀ = 1.0 in PBS and 0.5 ml were added the cover glass slides coated with Fn. The cover glass slides were incubated for two hours (microaerophilic for *C. jejuni* and at 37 °C for both *E. coli* and *C. jejuni*) and washed several times with PBS. Binding to Fn was visualised with crystal violet (0.1 %) staining.

6.3.3 Generation of *E. coli* BL21 (DE3) (Cj1349c)

Expression vector pET24b(+) (Kan^R), harbouring a full length Cj1349c gene, was PCR amplified from *C. jejuni* NCTC 11168 chromosomal DNA using primers Cj1349c-F1 (5'-ATATAGAGCTCCTCTTACTCGTGCAATGAGTGC) (*Sac*I site underlined) and Cj1349c-R1 (5'-ATATACTCGAGAATACTTTTAAAATTTGTATAATTTACAA) (*Xho*I site underlined). PCR was performed by 1 cycle of 4 min at 95 °C, 30 cycle of 1 min at 95 °C, 1 min at 55 °C and 1 min 72 °C, followed by 10 min at 72 °C. PCR products were verified on a 1 % agarose gel. The bands were cut out and purified by gel extraction kit (Qiagen), digested with BamH1 and EcoR1 and ligated into the equivalent sites of the pET24b(+) downstream of the T7 Lac promoter and the upstream of His₆ tag to produce a plasmid pET24b(+)-Cj1349c for protein overproduction. The ligation mixture was ethanol precipitated before electroporation into *E. coli* BL21(DE3).

6.3.4 Generation of 5 fragments of Cj1349c gene

Fragments of the Cj1349c gene were constructed in pET24b(+) as described for generation of *E. coli* BL21 (DE3) (Cj1349c). The 435 residues were divided into 5 fragments by the residues and primers. Fragment 1: 45-240 (primer F1 and R1), fragment 2: 45-320 (primer F1 and R2), fragment 3: 45-435 (primer F1 and R3), fragment 4: 150-320 (primer F2 and F3) and fragment 5: 320-435 (primer F3 and R3). *Sac*I restriction site was added forward primer and *Xho*I added reverse primer. Primer

for the generation (5'):

F1: ATATAGAGCTCTCTTACTCGTGCAATGAGTGC

F2: ATATAGAGCTCTAATGTGATTTTAGAGTCTTTAAAGC

F3: ATATAGAGCTCTCATAAACAAGAAGATCTTGTCGC

R1: ATATACTCGAGTTTGCCTTCAAAATCATCAAGC

R2: ATATACTCGAGAATTTTAAACTCGTTTAAAATAAAAATTTGC

R3: ATATACTCGAGAATACTTTTAAAATTTGTATAATTTACAA

6.3.5 Expression and purification of recombinant Cj1349c protein

An overnight pre-culture of *E. coli* BL21(DE3) containing the pET24b(+) plasmid (Novagen) was 50-fold diluted to inoculate 1000 ml LB medium containing 30 µg/ml kanamycin. The culture was incubated with shaking at 37 °C to an OD₆₀₀ of 0.5, then induced with 10 mM IPTG and incubated with shaking for 16 hours at 30 °C. After induction, cells were lysed on ice in 20 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 10 % glycerol) by addition of 1 mg/ml lysozyme followed by sonication. Lysates were cleared by centrifugation at 15,000xg for 30 min. His-tagged Cj1349c proteins were purified by nickel affinity chromatography using the Ni-NTA resin (Qiagen) equilibrated with lysis buffer. Cj1349c protein was eluted with 250 mM imidazole.

6.3.6 Fibronectin binding assay with purified protein

The wells of a 96-well plate were coated over night at 4 °C with either 1 µg of human Fn (Sigma) or BSA, which served as a negative control. The following day, the wells were rinsed with wash buffer (PBS containing 0.001 % Tween 20), blocked with 1% BSA for 2 h at 25 °C and rinsed once with wash buffer. To determine the Fn binding activity of the protein, twofold serial dilutions of Cj1349c, FlpA and CadF proteins were made in PBS, added to the wells, and incubated for 2 h at 25 °C. The CadF protein was used as a positive control for Fn binding affinity. After the wells were washed three times with wash buffer, a 1:1000 dilution of rabbit anti-His (Sigma) in incubation buffer was added to the wells and the plate was incubated at 25 °C for 1 h. The wells were then washed three times with wash buffer, a 1:5000 dilution of horseradish peroxidase labeled goat anti-His antibody (Sigma) diluted in PBS was added to the wells, and the

plate was incubated at 25 °C for 30 min. The wells were then washed, and bound antibodies were detected by addition of TMB substrate solution (Thermo Scientific). Binding was quantified by colorimetric detection at 492 nm.

6.3.7 Preparation of polyclonal antisera

Female New Zealand White rabbits were subcutaneously injected with 100 µg of purified Cj1349c protein in TiterMax Gold extract. A booster injection of 50 µg natively purified protein in Freund's incomplete adjuvant (Sigma) was given after 4 weeks. Blood was collected prior to the first and second immunizations, and 2 weeks after the second immunization. Sera were stored at -20 °C.

6.3.8 Immuno-fluorescence microscopy

Bacteria were grown microaerophilic 48 h at 37 °C. Then harvested at 4000 rpm and resuspended in 100 µl 4 % formalin in PBS. The cells were fixed 20 min at 4°C, followed by washing with PBS. Appropriate amount of fixed bacteria were added to single wells of a poly-L-lysine coated six-well glass slide (Novakemi Ab) and air-dried. The slide was washed in PBS for 10 min using a coplin jar. Primary polyclonal antibodies raised against Cj1349c in rabbit (see above) were diluted in PBS to a final ration of 1:100, 1:300 and 1:1000 and added to each well on the glass slide followed by incubation in a humid chamber for 45 min. After washing in PBS (in coplin jar), Cy3 conjugated secondary anti-rabbit antibodies (Alexa Fluor[®] 4594, goat anti-rabbit IgG, 2 mg/ml, Invitrogen) were diluted in a 1:500 ration and added to each well on the glass slide and incubated in a dark humid chamber. In the following steps, the glass slide was kept in the dark. After washing, the slide was air-dried and cover slips mounted in VECTASHIELD[®] Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Finally, the slide was examined with epifluorescence microscopy using the Cy3 spectrum of light for the fluorophore.

6.4 Results

6.4.1 Fibronectin binding assay with *E. coli* cells on glass cover slides

To study the ability of Cj1349c to bind Fn, the gene was over-expressed in *E. coli* and the ability of the *E. coli* to bind to Fn coated glass slides was tested. This resulted in

strong and reproducible binding to Fn of *E. coli* BL21(DE3) Cj1349c compared to *E. coli* BL21(DE3) (figure 6.1 A-C). FlpA (Cj1279c) was further included as a control and binding was confirmed but found weaker compared to Cj1349c.

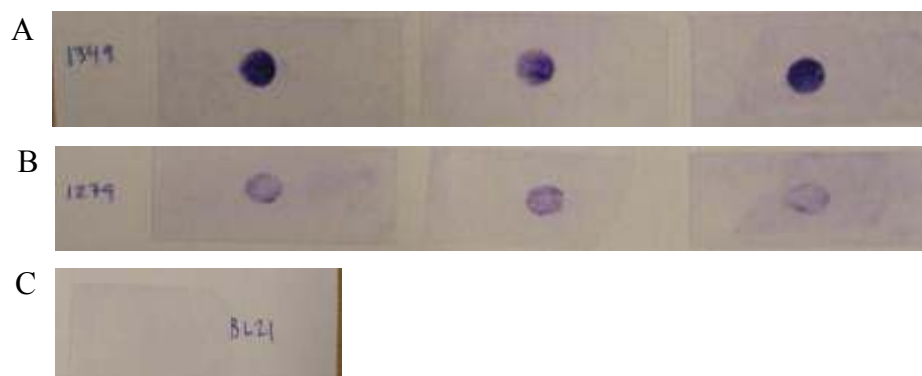


Figure 6.1. Fibronectin binding to glass slides. A. Glass slides coated with bovine fibronectin (40 μ l fibronectin (10 μ g/ml)) and added 0.5 ml overnight induced *E. coli* BL21(DE3) expressing Cj1349c. B. *E. coli* BL21(DE3) expressing FlpA (Cj1279c). C. *E. coli* BL21(DE3) with no insert.

6.4.2 Fibronectin binding assay with purified protein

Purified Cj1349c protein was used in an ELISA assay with microtiter plates coated with Fn. The results showed a relatively strong binding of purified Cj1349c protein to Fn. CadF was included as a control in this study exhibiting a high dose dependent binding compared to Cj1349c (figure 6.2). The binding of FlpA (Cj1279c) was found to be weaker which correlates with the results seen in the glass slide assay shown in figure 6.1.

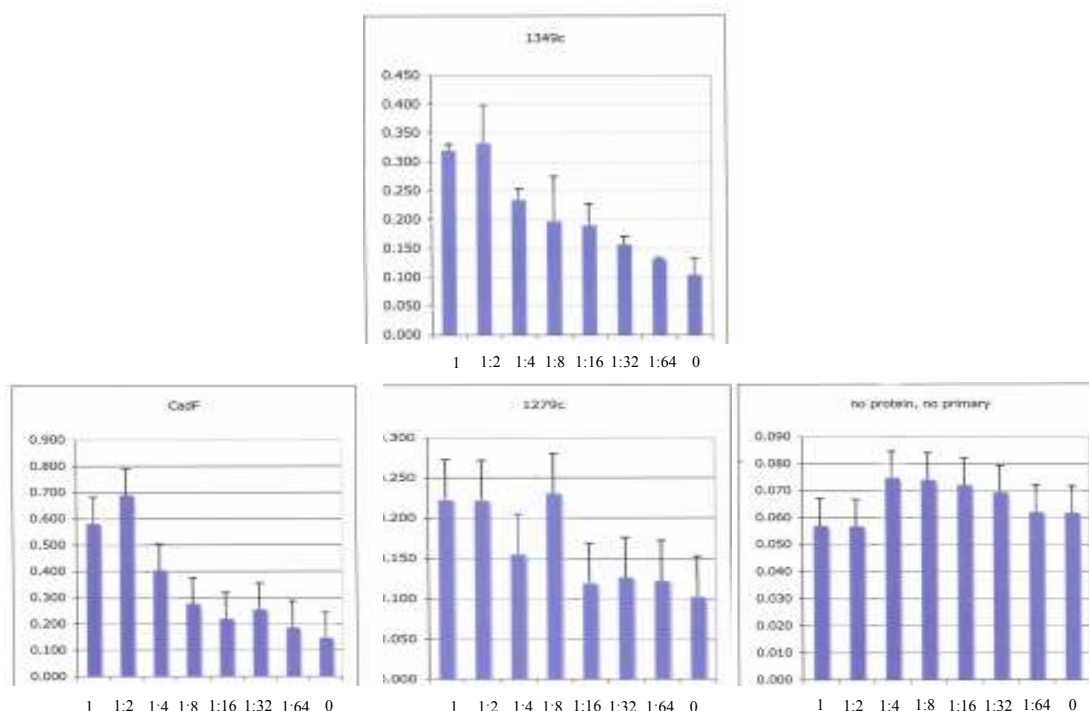


Figure 6.2. Fibronectin binding to purified protein. ELISA assay demonstrating the binding capacity of Cj1349c, CadF, FlpA (Cj1279c) and a control respectively. Y-axis presents OD₄₉₂ and X-axis the dilution row of Fn. As a control BSA was used. The binding capacity of Cj1349c was relatively strong and dose dependent compared to Cj1279c, but not as strong as for CadF.

5.4.3 Immuno-fluorescence microscopy

To test the specificity of the antiserum against Cj1349c made in rabbits, an immuno-fluorescence microscopy experiment was carried out. It was not possible to see any specific antibody binding using this assay.

6.5 Discussion and perspectives

Adherence to gastrointestinal cells and extracellular matrix components has previously been found crucial for *C. jejuni* for host interaction and disease. More particularly Fn, a component of the extracellular matrix, has been found to be an adhesion receptor for the *C. jejuni* proteins CadF and FlpA (Konkel et al., 1997; Konkel et al., 2010). Another *C. jejuni* protein has been annotated as a putative Fn binding protein and in this study we have shown its ability to bind Fn.

To assess the binding of Cj1349c to fibronectin, binding assays of whole bacterial to Fn

and an ELISA was used. The protein was expressed in *E. coli* in order to purify the protein to be used in ELISA. The assays showed consistency by revealing that Cj1349c binds to Fn and it binds relatively stronger than FlpA (Cj1279c). The assay was also attempted for *C. jejuni* NCTC 11168 but with no visual Fn binding. The binding of *C. jejuni* to Fn has previously been shown in *C. jejuni* F38011 (Konkel, M., personal communication) and the reasons for the failure in NCTC 11168 may be due to a lower expression level in NCTC 11168 or that it needs different growth or induction conditions. Both NCTC 11168 and F38011 are clinical isolates from patients diagnosed with *C. jejuni* but as previously reported, NCTC 11168 has changed phenotypes since the isolation, which could also be an explanation (Gaynor et al., 2004).

The ability of FlpA (Cj1279c) and Cj1349c to bind epithelial cells *in vitro* has been investigated previously (Flanagan et al., 2009). This study revealed a significant reduction of both the mutants of FlpA and Cj1349c to bind LMH chicken hepatocellular carcinoma epithelial cells. Furthermore, a chicken colonization experiment showed the FlpA mutant, but not the Cj1349c mutant, to be attenuated for colonization (Flanagan et al., 2009). CadF has likewise been shown to be important for chicken colonization (Ziprin et al., 1999). The different chicken colonization behaviour of the CadF, FlpA and Cj1349c mutants indicate the Fn binding proteins have different, and potentially host-specific functions, in the interaction with host cells. It can therefore not be excluded that Cj1349c is important for the virulence of *C. jejuni* in humans, and to pursue this hypothesis, investigations are in process to study the interactions of Cj1349c with human epithelial cells (results not shown). It is possible that expression of Cj1349c may need a host specific induction factor not taken into account in this experiment.

The domain on CadF with maximal Fn-binding activity has been localized to four amino acids (AA 134-137) consisting of the residues phenylalanine-arginine-leucine-serine (FRLS) (Konkel et al., 2005). We have constructed 5 *E. coli* clones each expressing a fragment of the Cj1349c protein and Fn-binding assays with these clones may reveal a novel *C. jejuni* binding site.

We have shown that the putative Fn binding protein, Cj1349c, binds to Fn in a glass

slide assay with *E. coli* expressing the protein and with purified protein in an ELISA assay. The binding domain of the protein to Fn and its biological importance is subject to ongoing research.

6.6 Acknowledgements

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7. Discussion and future perspectives

During this PhD study (chapter 3, appendix 2), we demonstrated that Danish *C. jejuni* isolates were genetically highly diverse by the use of MLST. Analysis of isolates from patients diagnosed with reactive arthritis were associated to many different clonal complexes, which suggest that this sequela rather involves differential expression of virulence factors. Analysis of isolates from GBS patients showed that these isolates were significantly over-represented in the ST-22 complex, which was also suggested previously (Dingle et al., 2001a). Previous studies, using MLEE typing, ribotyping, PFGE typing, flagellin gene typing and DNA Microarray found a significant diversity among GBS-related strains in contrast to our findings (Engberg et al., 2001; Leonard et al., 2004). A recent study, characterizing isolates from Bangladesh, identified the ST-403 complex to be correlated with GBS and further they found the ST-22 complex only in GBS isolates and not enteritis isolates (Islam et al., 2009). Penner heat-stabile (HS) serotype HS:19 has previously been associated with the development of GBS and the risk of developing GBS after infection with *C. jejuni* of serotype HS:19 was found to be several fold higher than with other serotypes (Nachamkin et al., 1998; Engberg et al., 2001). Islam et al (2009), in contrast, found the HS:23 strain to be dominant for GBS isolates in Bangladesh. Interestingly, the ST-403 complex belongs only to serotype HS:23 and ST-22 complex isolates belong only to the serotype HS:19. That is also the case for the isolates in this study, where five GBS isolates, belonging to ST-22 clonal complex, were Penner HS:19 serotyped (appendix 2). The study by Dingle et al (2001b) also had a overrepresentation of the serotype HS:19 in ST-22 clonal complex. These studies could indicate that HS:19 and ST-22 complex and HS:23 and ST-403, respectively, have clonal population structures. The studies further suggest that these groups might have unique virulence properties and that putative neuropathogenic *C. jejuni* serotypes and clonal complexes may circulate in different geographic areas. Also the LOS locus class A has been strongly associated with GBS outcome in several studies (Nachamkin et al., 2002; Islam et al., 2009) and it should also be kept in mind that differences in clinical outcome are likely to be attributable to differences in host background. It was proposed by the results from a family outbreak, in which one of three individuals with *C. jejuni* enteritis developed GBS and had a significant difference in the immune response towards LOS (Ang et al., 2000). Therefore, several factors

seem to be involved in the development of GBS, and the studies taken together could suggest an increased risk of GBS with certain clonal complexes perhaps in combination with a certain serotype, expression of the right LOS in combination with host responses. The fact that *C. jejuni* is naturally competent means that *C. jejuni* populations are subject to high rates of horizontal gene exchange with recombinational events contributing to a significant proportion of allelic diversity (Suerbaum et al., 2001; Schouls et al., 2003). This should also be taken into account when assessing the effectiveness of genotyping.

Although *C. jejuni* is the major cause of gastroenteritis worldwide little is known about its pathogenesis. Therefore, detection and identification of new virulence factors are crucial for this pathogen. The definition of a virulence factor can be divided into three; 1) true virulence genes (coding for factors or enzymes producing factors involved in all the processes of interaction with the host, responsible for the pathological damage during infection and absent in non-pathogens), 2) virulence associated genes (coding for factors or for enzymes producing factors that regulate expression of virulence genes or activate virulence factors or are required for the activity of true virulence factors) and finally, 3) virulence life-style genes (factors that enable the colonization of the host, evasion of the host immune system, intracellular survival or employ host-factors for the benefit of survival) (Wassenaar and Gaastra, 2001).

Virulence factors are often immunogenic as a result of the acquired immunesystem producing protective antibodies against virulence gene products (Wassenaar and Gaastra, 2001). We used this approach in our second study (manuscript 2, chapter 4), searching for both antigens in the purpose of vaccine development and in the search for new virulence genes. As vaccine candidates, the strategy is more straightforward. If the acquired immune system recognises the proteins, the product is specific and conserved for the targeted pathogen and easy to handle, express and isolate, then you have a possible good vaccine candidate. Several studies have been made and are still in progress in order to develop a vaccine against *C. jejuni* either for chickens thus indirectly reducing the number of human infections or for humans. Our study, described in manuscript 2, chapter 4, targeted for the latter. We identified 25 immunogenic

proteins in our screen. Five of them were tested as vaccine candidates by challenge experiments in mice; one of them, SSI-3, showed promising results by reducing invasion into the spleen and liver and some against colonization. Other studies have identified proteins for vaccine development, some associated with the *C. jejuni* surface and several of them are identical to proteins identified in this study. They are discussed in manuscript 2, chapter 4. Some of the identified antigenic proteins in this thesis have been predicted to be cytoplasmic proteins. This has also been shown in other studies. The cell binding factor, PEB4 (encoded by Cj0596) is immunogenic and located in the periplasma (Dubreuil et al., 1990) and is important for invasion and colonization (Rathbun et al., 2009). Also, the highly virulent lipoprotein *cjaA* (Cj0982c) has been identified to be localized in the inner membrane and further a promising vaccine candidate (Wyszynska et al., 2004, 2008). Prokhorova et al (2006) found several cytoplasmic proteins (ribosomal etc.) when identifying possible vaccine candidates. Immune responses directed against cytoplasmic proteins can be explained by a natural occurring lysis of the bacteria and thereby exposure of the proteins or that the protein is secreted. Another explanation may be the role of dendritic cells as antigen presenters, where the bacteria are probably broken down and presented by the dendritic cells. Other studies have also used *E. coli* libraries to identify immuno-reactive proteins. Connerton and Connerton (1999) used a lambda phage expression library and identified an integral membrane protein; *amaA* and Galindo et al (2001) identified, by the use of a cosmid library, an 80-kDa antigen. The method has also been applied to other pathogens; Padmalayam et al (2000) identified an immunogenic lipoprotein of *Bartonella bacilliformis*, and Werner et al (2006) identified an immuno-dominant antigen of *Bartonella henselae* by constructing cosmid libraries.

Even though the argument that virulence factors are often immunogenic cannot be directly reversed (Wassenaar and Gaastra, 2001), we tested if our immunogenic proteins could be associated with virulence. It cannot be reversed because some immunogenic proteins are solely structural components of the bacterial cell. Chaperons, for example, can raise an antibody response and have adherence properties to epithelial cells (Brøndsted et al., 2005) but their function in stress repair and folding is of greater significance than their virulence properties (Wassenaar and Gaastra, 2001). On the other

hand, proteins needed for infection are most likely expressed at high levels during infection and are therefore, possible targets for the immune system. Some of the immunogenic proteins, identified in manuscript 2, chapter 4, were studied in an adhesion assay in their natural host, *C. jejuni* NCTC11168 as knock-out mutants and one protein seemed particularly interesting (Cj0034c). The protein was further analysed for altered motility, autoagglutination, biofilm formation, and serum resistance but with no altered phenotypes. We further studied the protein in an animal mouse model but neither the *C. jejuni* wildtype nor the mutant succeeded in colonization (data not shown). In the studies described in both manuscript 2 and 3 (chapter 4 and 5), we have faced one of the probably most common obstacles, when studying *C. jejuni* virulence factors; the lack of a solid and reproducible animal model that could prove the association between the proteins and adhesion, colonisation and possible virulence. Another study has identified an adhesion protein without proving its role in an animal model (Jin et al., 2001). The JlpA (jejuni lipoprotein A) was seen to be surface-exposed but not immunogenic, mutants of this protein had reduced adherence to HEp-2 cells and antibodies against JlpA inhibited the binding in a dose dependent manner. These results taken together demonstrated the JlpA protein to be an adhesion factor. To show that protein Cj0034c was an adhesion factor, we should complement the knock-out mutant and show its re-established binding to INT407 epithelial cell. Further, we could produce antibodies against the protein and examine a possible binding inhibition to the epithelial cell. Furthermore, the localisation of the protein and target molecule on the host cells would be of great interest. Several other identified genes in manuscript 2 and 3 could be important for *C. jejuni* interaction with the human host or for survival strategies such as biofilm formation, and further studies will be made in order to confirm the results.

Flagellin has previously been shown to be a strong antigen and also important for autoagglutination (Cawthraw et al., 1994; Guerry et al., 2006), therefore it is striking that it has not been identified in either the antigen or the autoagglutination screening. An explanation can be how the expression library was constructed. A pool of the original vectors from the *E. coli* strains was purified and then transformed to the BL21(DE3) expression strain. During this purification step the flagellin gene, among other, could be lost or it could have been lost during the transformation step into *E. coli* BL21.

In the study, outlined in manuscript 4, chapter 6, we used another approach in order to identify proteins important for host interaction. A protein predicted to be an adherence factor with a known target molecule was studied. We showed that the protein, expressed by the Cj1349c gene, did bind to fibronectin when expressed in *E. coli* and when purified. Binding to fibronectin is important for *C. jejuni* and adhesion to the host. The importance of CadF and FlpA has been proved by adherence to human epithelial cells and for colonization in chickens (Konkel et al. 1997; Flanagan et al., 2009). Cj1349c does also bind to human epithelial cells but has no effect on chicken colonization. It can still not be excluded that Cj1349c is important for human host interaction, by adhesion and a possible activation of integrins, rearrangement of cytoskeleton and thereby possible invasion of the bacteria. We have produced antibodies against Cj1349c but as there was no binding in fluorescence microscopy we speculate if the serum is specific enough. The lack of binding could also be caused by a low expression level of Cj1349c on *C. jejuni* and we need to confirm our speculation by a western blot with purified protein and the serum with antibodies raised against purified Cj1349c. It is also possible that the transcription of the Cj1349c protein are triggered by different culture conditions and it has been shown that the protein was upregulated when *C. jejuni* was cultured with the bile acid deoxycholate (Malik-Kale et al., 2008).

Even though we have contributed to the knowledge regarding *C. jejuni* and host interactions by identifying novel putative surface proteins, finding a possible vaccine candidate and verifying a new fibronectin binding protein, further analysis needs to be done on these proteins to verify their exact role in the *C. jejuni* pathogenesis. Regarding the pathogenesis of *C. jejuni* in general, much work is also needed to identify the reasons why this bacterium is so successful when it comes to human infections despite its relatively demanding growth requirements. Probably we need to move focus from known virulence factors and methods utilised assessing pathogenesis in other bacteria and focus on human host responses and novel methods. Furthermore, genomic sequencing and comparison of *C. jejuni* strains will probably contribute with important insight into the *C. jejuni* pathogenesis.

8. Overall conclusion

In conclusion, the experimental studies included in this thesis have shown a possible correlation between a MLST clonal complex and the severe clinical post infection, Guillain-Barré syndrome. The method was useful to study the population clonality of *C. jejuni*, and un-typeable strains were not identified. We identified several immunogenic proteins of *C. jejuni* of which one is a possible vaccine candidate and one may be associated to cell adherence in the human host. We also found proteins involved in survival mechanisms, such as biofilm-formation, which could be of great interest to further studies. We showed that screening of an *E. coli* library is a very effective way in order to identify new antigens or surface exposed proteins. Additionally, we showed that the putative fibronectin binding protein encoded by Cj1349c, indeed did bind to fibronectin and could be of significant importance to human host interactions.

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10. Appendices

10.1 Appendix 1: Conference presentations

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Identification of immunogenic *Campylobacter jejuni* surface structures.

Oral presentation at Second Symposium on Food Associated Pathogens, Grythyttan, Sweden, August 2010.

Nielsen, L. N., Larsson, C., Krogfelt, K. A., Ingmer, H., Konkelt, M. Identification of a fibronectin binding protein of *Campylobacter jejuni*.

Poster presentation at Symposium on Food Microbiology, no. 8, Helsingør, Denmark, June 2010.

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Screening for Immunogenic *Campylobacter jejuni* Proteins.

Poster presentation at 108th General meeting American Society for Microbiology, Boston, USA, June 2008.

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Identification of immunogenic *Campylobacter jejuni* surface structures.

Oral presentation at Symposium on Food Microbiology, no. 6, Helsingør, Denmark, May 2008.

Nielsen, L. N., Sheppard, S. K., McCarthy, N., Krogfelt, K. A., Ingmer, H. Multilocus sequence typing of *Campylobacter jejuni* isolates from Denmark.

Poster presentation at International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, no. 14, Rotterdam, Holland, September 2007.

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Screening for Immunogenic *Campylobacter jejuni* Proteins.

Poster presentation at Experimental workshop on: Signal Transduction in Host-Bacterial Interactions, Jerusalem, Israel, October 2007.

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Screening for Immunogenic *Campylobacter jejuni* Proteins.

Poster presentation at International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms, no. 14, Rotterdam, Holland, September 2007.

Nielsen, L. N., Vegge, C. S., Krogfelt, K. A., Ingmer, H. Screening for Immunogenic *Campylobacter jejuni* Proteins.

Poster presentation at Symposium on Food Microbiology, no. 5, Helsingør, Denmark, May 2007.

10.2 Appendix 2: Paper 1

MLST clustering of *Campylobacter jejuni* isolates from Patients with Gastroenteritis, Reactive arthritis and Guillain-Barré Syndrome.

Lene N. Nielsen^{1,2}, Samuel S. Sheppard³, Noel D. McCarthy³, Martin C. Maiden³, Hanne Ingmer², Karen A. Krogfelt¹ **1** Statens Serum Institut, Department of Microbial Surveillance and Research, Copenhagen, Denmark. **2** Department for Veterinary Disease Biology, Faculty of life Sciences, University of Copenhagen, Denmark. **3** Department of Zoology, University of Oxford, United Kingdom.

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Supplementary to paper:

A number of the Guillain-Barré isolates have been Penner serotyped in a previous study (Nachamkin et al., 2002).

Original strain	This study	Serotype	Clonal complex
KB3449	1575	HS:19	22
OH4382	1576	HS:19	22
INP7	1577	HS:19	22
HB9635	1578	ND	354
INP59	1579	HS:41	42
HB96-43	1580	HS:19	22
HB95-29	1581	HS:19	22
INP21	1582	HS41	42